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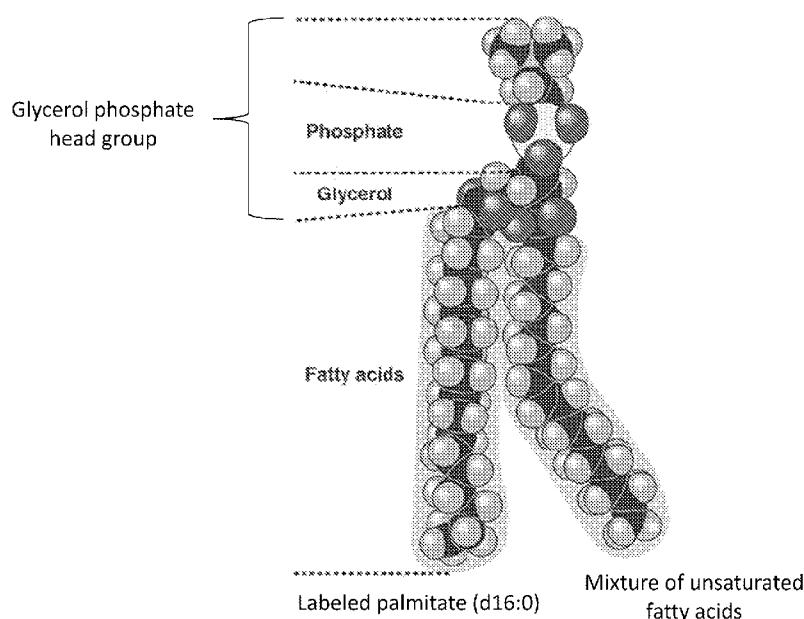
(57) Abstract: Methods are provided for synthesizing mixtures of lipids that are representative of the structural diversity of the lipids present in samples of interest. The complex mixtures of lipids produced according to the methods of the present disclosure can be used as internal standards for detecting and quantifying the lipids in samples of interest. Kits including the internal standards and instructions for their use in the detection and quantification of lipids in samples of interest are also provided.

Glycerol phosphate head group

Phosphate

Glycerol

Fatty acids

Labeled palmitate (d16:0)   Mixture of unsaturated fatty acids

FIG. 1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

METHODS, COMPOSITIONS, AND KITS FOR ANALYSIS OF STRUCTURALLY DIVERSE COMPLEX LIPIDS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001]   This application claims the benefit of U.S. provisional patent application number 62/031,099 filed July 30, 2014, the disclosure of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002]   The presently disclosed subject matter relates to methods, compositions, and kits for synthesizing and using mixtures of complex lipids as internal standards for analysis of biological samples and other samples containing mixtures of complex lipids.

BACKGROUND

[0003]   Using an internal standard to calibrate the concentration of a target compound in a sample is a well-accepted method of quantification. In short, a compound is added that is not present in the sample to be tested that satisfies two criteria: (1) that the internal standard act similarly to the target compound at all stages of sample preparation (e.g. extraction, chromatography, etc) and that (2) it is distinguishable from the target compound by the detection method of choice. Thus, if one knows the concentration of the internal standard added to the sample, and the relative response of the detector (e.g. peak area) to both the target compound and the internal standard, one can calculate the amount of target compound that must have been in the original sample.

[0004]   Lipids in biological samples are problematic targets for quantification by internal standard because they have a diverse array of chemical properties, even within a given lipid class. The lipids also have combinatorial complexity, for example, several of many potential fatty acids are acylated onto one of a dozen or so backbones. Thus a small number of internal standards cannot meet the first of the above two criteria. Generally, analysts use few internal standard compounds, and most often choose only a single compound to represent a broad class of

lipid (e.g., phospholipids, neutral lipids) to use as an internal standard. The choice of the standard for the broad class has historically related more to the absence of the compound in biological samples and the ease of synthesis of the synthetic compound than to the similarity of the standard to the chemical properties of the target analytes. Most lipid internal standards are constructed from a single odd-chain or short-chain fatty acid (e.g. PC12:0/12:0 or TAG17:0/17:0/17:0). Most mixtures of internal standards use a single internal standard per broad lipid class. However, current approaches for developing internal standards are limited because: 1) short chain fatty acids or completely saturated fatty acid internal standards do not act like the target compounds in the assay and 2) it is difficult to represent the combinatorial complexity of lipids using simple internal standard strategies.

[0005]   There is a need for a structurally diverse set of standards with appropriate chemical properties for each lipid class for use as internal standards. Such a set of internal standards provides broad chemical diversity and thus better coverage for analysis.

SUMMARY

[0006]   Described herein are methods, kits, and compositions relating to internal standards for use in mass spectrometry analysis. Complex lipids are classes of lipids with a diverse array of fatty acids attached to them. Methods of synthesizing and using compositions and kits that include mixtures of lipid molecules as internal standards with a sufficiently diverse composition to effectively represent the fatty acids of the complex lipid class(es) in samples of interest are provided herein . The internal standards are synthesized to contain the characteristics described herein. The synthesized internal standards may then be packaged in a kit and used for lipid analysis. The lipid internal standards described herein may be used in any application including, for example, mass spectrometry.

[0007]   In one embodiment of the present disclosure, a method is provided for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising: attaching an isotopically-labeled fatty acid at a first position on a lipid backbone through an acylation reaction for a lipid class having at least two acyl groups; and attaching a mixture of at least two different fatty acids to the lipid backbone at a separate position through an acylation reaction, wherein the mixture of fatty acids is representative of the fatty acids that

occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0008]   In one embodiment of the present disclosure, a method is provided for synthesizing one or more mixture of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising: attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0009]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups; and a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0010]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having one or more isotopic labels, wherein the lipid backbone is for a lipid class having at least one acyl group; and a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present

at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0011]   In one embodiment of the present disclosure, a kit is provided comprising: i) one or more mixtures of lipid molecules for use as an internal standard, wherein each mixture of lipid molecules is representative of the composition of lipid molecular species present in each of one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone and a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups, or a lipid backbone having one or more isotopic labels and a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the lipid class in the sample of interest; and ii) instructions for using the one or more mixtures of lipid molecules as the internal standard for one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0012]   In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, comprising: adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, wherein each mixture of lipid molecules comprises i) a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups; and ii) a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest; and one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in

the sample of interest by using the representative mixture of lipid molecules as an internal standard.

[0013]   In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, comprising: adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, wherein each mixture of lipid molecules comprises i) a lipid backbone having one or more isotopic labels, wherein the lipid backbone is for a lipid class having at least one acyl group; and ii) a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest by using the representative mixture of lipid molecules as an internal standard.

[0014]   In one embodiment of the present disclosure, a method is provided for synthesizing one or more mixtures of lipid molecules for use as an internal standard representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising one or more of: attaching a mixture of at least two different isotopically-labeled fatty acids to a lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; or attaching a single isotopically-labeled fatty acid to a lipid backbone at a first position through an acylation reaction for a lipid class having at least two fatty acids and attaching a mixture of at least two different fatty acids to a separate position on the lipid backbone through an acylation reaction, wherein the mixture of the at least two different fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0015]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of: a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids, wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0016]   In one embodiment of the present disclosure, a kit is provided comprising: i) one or more mixtures of lipid molecules for use as an internal standard, wherein each of the one or more mixtures of lipid molecules is representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of: a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups, wherein the mixture of at least two different fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species

present in the corresponding lipid class in the sample of interest; and ii) instructions for using the one or more mixtures of lipid molecules as the internal standard for one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest.

[0017]   In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, the method comprising: i) adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, the composition comprising one or more of: a) a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; b) an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or c) a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids, wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the lipid class in the sample of interest; and ii) one of detecting and quantifying the lipid molecular species present in each of the one or more corresponding lipid classes in the sample of interest by using the composition having the one or more representative mixtures of lipid molecules as an internal standard.

DESCRIPTION OF DRAWINGS

[0018]   FIG. 1 is a schematic of a phospholipid showing a general strategy for producing phospholipid internal standards according to one or more embodiments of the present disclosure.

[0019]   FIG. 2A-2C show the structure of phosphatidylcholine (PC) internal standards according to one or more embodiments of the present disclosure. (A) deuterated palmitate (16:0) in the sn-1 position and the fatty acid "R" in the sn-2 position, (B) the exemplary fatty acid

oleate (18:1n9) in the sn-2 position, and (**C**) ten exemplary fatty acids for acylating into the sn-2 position.

[0020]   **FIG. 3** shows the structure of a lysophosphatidylcholine (LPC) internal standard according to one or more embodiments of the present disclosure.

[0021]   **FIG. 4A-4C** show the structure of phosphatidylethanolamine (PE) internal standards according to one or more embodiments of the present disclosure. (**A**) deuterated octadecanoic acid (18:0) in the sn-1 position and the fatty acid "R" in the sn-2 position (**B**) the exemplary fatty acid oleate (18:1n9) in the sn-2 position (**C**) eight exemplary fatty acids for acylating into the sn-2 position.

[0022]   **FIG. 5** shows the structure of a lysophosphatidylethanolamine internal standard according to one or more embodiments of the present disclosure.

[0023]   **FIG. 6A-6C** show the structure of sphingomyelin internal standards according to one or more embodiments of the present disclosure. (**A**) deuterium-labeled sphingosine at the first position, and the fatty acid "R" in the sn-2 position, (**B**) the exemplary fatty acid palmitate (16:0) in the sn-2 position, and (**C**) four exemplary fatty acids for acylating into the sn-2 position.

[0024]   **FIG. 7A-7C** show the structure of triacylglycerol internal standards according to one or more embodiments of the present disclosure . (**A**) deuterium-labeled palmitate (16:0) at the sn-1 position, oleate (18:1n9) at the sn-2 position, and the fatty acid "R" in the sn-3 position, (**B**) the exemplary fatty acid palmitate (16:0) in the sn-3 position, and (**C**) eight exemplary fatty acids for acylating into the sn-3 position.

[0025]   **FIG. 8A-8C** show the structure of diacylglycerol internal standards according to one or more embodiments of the present disclosure. (**A**) deuterium-labeled palmitate (16:0) at the sn-1 position, and the fatty acid "R" in the sn-2 position, (**B**) the exemplary fatty acid palmitate (16:0) in the sn-2 position, and (**C**) eight exemplary fatty acids for acylating into the sn-2 position.

[0026]   **FIG. 9A-9C** show the structure of cholesteryl ester internal standards according to one or more embodiments of the present disclosure. (**A**) deuterium labels at the n6 position, and the fatty acid "R" acylated to the hydroxyl group, (**B**) the exemplary fatty acid linoleate (18:2n6) acylated to the hydroxyl group, and (**C**) eight exemplary fatty acids for use in acylating to the hydroxyl group.

[0027]   FIG. 10A-10B show the structures of exemplary free fatty acids according to one or more embodiments of the present disclosure. (A) deuterated palmitate, and (B) 17:1n7 for the internal standard mixture.

[0028]   FIG. 11 is a graph showing the actual concentrations of the indicated phosphatidylcholine species in an unlabeled internal standard mix sample compared to the concentrations calculated using a traditional internal standard (IS) PC17:0/17:0 and an exemplary IS mix according to one or more embodiments of the present disclosure. Concentrations are displayed in mole % composition.

[0029]   FIG. 12 is a graph showing the concentration calculated using the internal standard mixture and the precision of the calculations (in %CV) for 15 human serum samples according to one or more embodiments of the present disclosure.

[0030]   FIG. 13 shows box plots of the total concentration of the cholesteryl ester (CE) lipid class, calculated using the internal standard mixture, for 15 human serum samples run in triplicate according to one or more embodiments of the present disclosure.

[0031]   FIG. 14 shows box plots of the total concentration of the triacylglycerol (TAG) lipid class, calculated using the internal standard mixture, for 15 human serum samples run in triplicate according to one or more embodiments of the present disclosure.

[0032]   FIG. 15A-15C are plots showing the total concentrations of (A) cholesteryl ester (CE); (B) phosphatidylcholine (PC); and (C) phosphatidylethanolamine (PE) lipid classes calculated using a single internal standard and multiple internal standards according to one or more embodiments of the present disclosure.

[0033]   FIG. 16 is a plot showing the relative amount of the 22 fatty acids measured in the cholesteryl ester (CE) lipid class calculated using a single internal standard and multiple internal standards according to one or more embodiments of the present disclosure.

[0034]   FIG. 17A-17D show the concentrations of exemplary polyunsaturated fatty acids in the phosphatidylcholine (PC) or cholesteryl ester (CE) lipid classes calculated using a single internal standard and multiple internal standards according to one or more embodiments of the present disclosure. (A) PC20:5; (B); PC22:6 (C) CE40:4; and (D) CE20:5.

DETAILED DESCRIPTION

[0035] For the purposes of promoting an understanding of the principles of the present disclosure, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the disclosure is thereby intended, such alteration and further modifications of the disclosure as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the disclosure relates.

[0036] Lipids are problematic targets for quantification by internal standards because they have a diverse array of chemical properties, even within a given lipid class. The lipids also have combinatorial complexity, for example, several of many potential fatty acids are acylated onto one of a dozen or so backbones. Thus a small number of internal standards cannot meet the first of the above two criteria. What is needed is a way to make a set of internal standards that (1) represents the chemical diversity of complex lipids and (2) creates a mixture of these standards in a relative abundance, representing the expected composition of lipids in a sample, which makes the compounds useful as internal standards. The presently disclosed subject matter provides methods and compositions for internal standards that are representative of the chemical diversity of complex lipids.

[0037] Described herein are reagents and kits for a structurally diverse set of lipid standards with appropriate chemical properties and/or analytical properties for each lipid class. With respect to the nomenclature for fatty acid "lipid metabolites" (or otherwise referred to herein as "lipid molecules") used herein, fatty acids labeled with a prefix "CE", "DG", "FA", "PC", "PE", "LPC", "LPE", "O-PC", "P-PE", "SM", "TG", or "CER" refer to the indicated fatty acids present within cholesteryl esters, diacylglycerols (diglycerides), free fatty acids, phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines, lysophosphatidylethanolamines, 1-ether linked phosphatidylcholines, 1-vinyl ether linked phosphatidylethanolamines (plasmalogens), sphingomyelins, triacylglycerols (triglycerides), and ceramides, respectively, in a sample. In some embodiments, the indicated fatty acid components are quantified as a proportion of total fatty acids within the lipid class indicated by the prefix. References to fatty acids without a prefix or other indication of a particular lipid class generally indicate fatty acids present within total lipids in a sample. The term "LC" following a prefix "CE", "DG", "FA", "PC", "PE", "LPC", "LPE", "O-PC", "P-PE", "SM", "TG", or "CER" refers

to the amount of the total lipid class indicated by the prefix in the sample (e.g., the concentration of lipids of that class expressed as nMoles per gram of serum or plasma). For example, with respect to a measurement taken from plasma or serum, in some embodiments, the abbreviation "PC 18:2n6" indicates the percentage of plasma or serum phosphatidylcholine comprised of linoleic acid (18:2n6), and the term "TGLC" indicates the absolute amount (e.g., in nMoles per gram) of triglyceride present in plasma or serum. "MUFA", "PUFA", and "SFA" refer to monounsaturated fatty acid, polyunsaturated fatty acid, and saturated fatty acid, respectively.

[0038]   As used herein, "lipid backbone" refers to the portion of the lipid molecule that excludes the fatty acid groups or acyl groups. As used herein, the terms "fatty acids" and "acyl groups" are used interchangeably. As used herein, "lipid molecular species" or "molecular species" refers to a lipid molecule comprised of a specific fatty acid or acyl group attached to a specific lipid backbone (e.g., PC 18:2n6, CE 16:1n6, etc.).

[0039]   As used herein, "isotopically labeled" refers to an internal standard or any portion of an internal standard marked for determination using any suitable moiety (e.g., deuterium (^{2}H, ^{13}C, ^{15}N). Any atom or any number of atoms of the internal standard may be labeled with the isotope. For example, the isotope may be deuterium, and the internal standard may contain 9 deuterium atoms. In another example, the isotope may be deuterium and the internal standard may contain 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, or 31 deuterium atoms.

[0040]   One use of the methods described herein is for calibrating or aiding in calibrating mass spectrometry instruments or multiple mass spectrometry instruments run in tandem (i.e., a "platform"). In one example, two types of calibration are required: one for lipid class concentrations and one for fatty acid composition of the class. The results of the platform calibration may be computationally calibrated using either the control samples or comparison to a quantitative database of results.

[0041]   The methods described herein to generate an internal standard mixture may be applied to any lipid or class of lipid The term "lipid class" as used herein is meant to refer to a class of lipid including, for example, triacylglycerols, diacylglycerols, cholesteryl esters, free fatty acids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, lysophosphatidylserine, phosphatidylinositol, lysophosphatidylinositol, phosphatidylglycerol, lysophosphatidylglycerol, phosphatidic acid,

lysophosphatidic acid, CDP-diacylglycerol, lyso CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, wax diesters, and 1-monoacylglycerol. The triacylglycerols, diacylglycerols, cholesteryl esters, free fatty acids are broadly as neutral lipids. The phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, lysophosphatidylserine, phosphatidylinositol, lysophosphatidylinositol, phosphatidylglycerol, lysophosphatidylglycerol, phosphatidic acid, lysophosphatidic acid, CDP-diacylglycerol, lyso CDP-diacylglycerol are broadly as phospholipids.

[0042]   The internal standard mixture may include a phospholipid wherein each phospholipid head group (e.g., PC, PE, SM) contains a) an isotopically-labeled fatty acid wherein the isotopically-labeled fatty acid is at the sn-1 or sn-2 position; and b) an acylated mixture of fatty acids, wherein the acylated mixture of fatty acids is at a separate position (i.e., the position not occupied by the isotopically-labeled fatty acid), and wherein the acylated mixture of fatty acids approximates the concentration of fatty acids in the target complex lipid.

[0043]   In one example, the isotopically-labeled fatty acid is a saturated fatty acid, and the acylated mixture of fatty acids is a pre-defined mixture of unsaturated and polyunsaturated fatty acids. In one example, the isotopically-labeled saturated fatty acid is palmitate (16:0) at the sn-1 position, and the acylated mixture of unsaturated and polyunsaturated fatty acids is a pre-defined mixture at the sn-2 position. In one example, the acylated mixture is comprised of unsaturated fatty acids. In one example, the phospholipid is lysophosphatidylcholine, and the phospholipid head group contains isotopically-labeled palmitate (16:0) at the sn-1 position. In another example, the phospholipid is lysophosphatidylethanolamine, and the phospholipid head group contains isotopically-labeled stearate (18:0) at the sn-1 position. The method for producing the mixture of fatty acids is as follows: 1) determine the concentration of fatty acids in the given sample type, the concentration may be already known or may be determined by fatty acid composition analysis; 2) select a fatty acid from one or more representative degrees of unsaturation (e.g., monoene, diene, triene, tetraene, pentaene, hexaene), wherein the most abundant fatty acid in the lipid class is selected; 3) based upon the concentration of the fatty acid in the sample type, assign each fatty acid in the mixture as "high" or "low" abundance; 4) assign

a percentage value of the mixture corresponding to the high and low abundance (e.g., a fatty acid with "high" abundance would comprise 20% of the mixture and a fatty acid with "low" abundance would comprise 5%); and 5) for the remaining percentage of the mixture, determine the most abundant fatty acids among unsaturated fatty acids and assign a high or low abundance as described above to complete the mixture.

[0044]   The internal standard mixture may include a phospholipid wherein the head group of each phospholipid class (e.g., PC, PE, SM) contains a) an unlabeled fatty acid, wherein the unlabeled fatty acid is at the sn-1 or sn-2 position; and b) a MUFA or PUFA, wherein the MUFA or PUFA is at the position not occupied by the unlabeled fatty acid. In one example, the head group contains a) an unlabeled odd chain saturated fatty acid, wherein the unlabeled odd chain saturated fatty acid is at the sn-1position; and b) an odd chain MUFA or PUFA, wherein the odd chain MUFA or PUFA is at the sn-2 position.

[0045]   The internal standard mixture may include neutral lipids wherein the neutral lipids include mixtures of isotopically-labeled MUFA or odd-chain MUFA (e.g., 17:1, 19:1, etc.).

[0046]   In some embodiments, a quantitative fatty acid analysis may determine the exact composition of the internal standard mixture. In one example for phospholipids, the sn-1 position of each phospholipid head group is completely labeled with a single deuterated saturated fatty acid (e.g. d16:0), therefore the remainder of the fatty acids are present in the sn-2 position. The equation is as follows: $1 = A/X + B/X + C/X$, etc., where X is the percent of the single deuterated saturated fatty acid in the mixture; where A, B, C, etc. are fatty acids in the mixture; and where the percent of the fatty acids A, B, C, etc. in the internal standard mixture is known relative to X. Further, A/X=amount of the mixture comprised of A. For example, if the composition of the internal standard mixture for PC as determined by FAME analysis is: d16:0 - 50%, 18:1n9 - 10%, 18:2n6 -20%, and 20:4n6 - 20%, then $1 = (0.1/0.5) + (0.2/0.5) + (0.2/0.5)$ and $0.1/0.5=0.2$; $0.2/.5=0.4$; and $0.2/0.5=0.4$, then the mixture is comprised of 20% PCd16:0/18:1n9, 40% PCd16:0/18:2n6, and 40% PCd16:0/20:4n6.

[0047]   A free fatty acid is one type of neutral lipid that may comprise the internal standard mixture. The free fatty acid may be any free fatty acid including, for example, a single odd-chain fatty acid (e.g. 17:1, 17:2, 17:0) and/or an isotopically-labeled fatty acid (e.g.16:0, 16:1, 17:0). The free fatty acid may be a MUFA or the fatty acid may be a PUFA.

[0048]   A cholesteryl ester is one type of neutral lipid that may comprise the internal standard mixture. The cholesteryl ester may be any cholesteryl ester including, for example a cholesteryl ester comprised of a labeled cholesterol molecule, esterified to a mixture of fatty acids representing the expected composition of cholesteryl esters in the sample (see the Table 1).

[0049]   Diacylglycerols are one type of neutral lipid that may comprise the internal standard mixture. The diacylglycerol may be any diacylglycerol including, for example, a single odd-chain fatty acid or an isotopically-labeled fatty acid. The fatty acid chains of the diacylglycerol may contain a) an isotopically-labeled fatty acid wherein the isotopically labeled fatty acid is at the sn-1 or sn-2 position (a first position); and b) an acylated mixture of fatty acids, wherein the acylated mixture of fatty acids is at a separate position (i.e., the position not occupied by the isotopically-labeled fatty acid). In one example, the isotopically-labeled fatty acid is a saturated fatty acid. In one example, the isotopically-labeled fatty acid is palmitate (16:0) at the first position, and the acylated mixture of fatty acids is at the separate position and may comprise 16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, and 22:6n3. In one example, the isotopically-labeled fatty acid is palmitate (16:0) at the first position and the acylated mixture of fatty acids at the separate position is a mixture of unsaturated and polyunsaturated fatty acids. . In another example, the fatty acid chains of the diacylglycerol in the internal standard mixture may contain a) an unlabeled odd chain saturated fatty acid, wherein the unlabeled odd chain saturated fatty acid is at the first position; and b) an odd chain MUFA or PUFA, wherein the odd chain MUFA or PUFA is at the separate position. The diacylglycerol in the internal standard mixture may use multiple homogenous internal standards (e.g. DG17:0/17:0 and DG17:1/17:1).

[0050]   A triacylglycerol is one type of neutral lipid that may comprise the internal standard mixture. The triacylglycerol may be any triacylglycerol including, for example, a mixture of an odd-chain fatty acid and/or an isotopically-labeled fatty acid to make a mixed standard. The fatty acid chains of the triacylglycerol may contain a) a labeled fatty acid at one position; b) an unlabeled fatty acid at a separate position; and c) an acylated mixture of fatty acids at another separate position. In one example, the labeled fatty acid may be a saturated fatty acid, and the unlabeled fatty acid may be an unsaturated fatty acid. In one example, the label may be a deuterium label. In one example, the fatty acid chains of the triacylglycerol in the internal standard mixture contain a) isotopically-labeled palmitate (16:0) at one position (e.g., the sn-1 position); b) oleate (18:1n9) at a separate, or second, position (e.g. the sn-2 position); and c) an

acylated mixture of fatty acids at another separate, or third, position (e.g., the sn-3 position). The acylated mixture of fatty acids at the third (e.g., sn-3) position may comprise, for example, 16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6 and 22:6n3.

[0051]   The internal standard mixture may comprise: a) phosphatidylcholine lipid class with isotopically-labeled palmitate (16:0) in the sn-1 position and a mixture of MUFA and PUFA in the sn-2 position; b) phosphatidylethanolamine lipid class with isotopically-labeled palmitate (16:0) or stearate (18:0) in the sn-1 position, and a mixture of MUFA and PUFA in the sn-2 position; c) lysophosphatidylcholine lipid class with isotopically-labeled palmitate (16:0) in the sn-1 position; d) lysophosphatidylethanolamine lipid class with isotopically-labeled palmitate (16:0) or stearate (18:0) in the sn-1 position; e) sphingomyelin lipid class with an isotopically-labeled sphingoid backbone and a mixture of fatty acids in the sn-2 position; f) triacylglycerol lipid class with isotopically-labeled palmitate in the sn-1 position, unlabeled oleate (or other fatty acid) in the sn-2 position, and a mixture of fatty acids in the sn-3 position; g) cholesteryl ester lipid class with isotopically-labeled cholesterol headgroup acylated to a mixture of fatty acids; h) diacylglycerol lipid class with isotopically-labeled palmitate (16:0) in the sn-1 position and a mixture of fatty acids in the sn-2 position; i) free fatty acid lipid class with isotopically-labeled fatty acids or 15:1 or 17:1; and/or j) ceramide lipid classes with an isotopically-labeled sphingolipid backbone and a fatty acid mixture appropriate to the ceramide class.

[0052]   In one example, the ceramide lipid class may be comprised of the following sphingolipid backbones: sphingosine, dihydrosphingosine, phytosphingosine, or 6-hydroxysphingosine.

[0053]   The reagents described herein may be combined as an article of manufacture, for example, as a kit.

[0054]   Complex lipids are categorized by classes; each class is defined by the head group moiety on the lipid (e.g. PC has a phosphocholine headgroup, CE has a cholesterol headgroup). Within a lipid class, there are many molecular species that are defined by the fatty acids that are linked to the headgroup. Because fatty acids have diverse chemical structures, each lipid class is comprised of a large number of diverse components which results in distinct lipid molecular species. To quantify a lipid class,
one needs to quantify each of these molecular species and sum them accurately.

[0055]   In contrast to traditional lipidomic strategies which typically use only a single internal standard per broad lipid class (e.g., phospholipids), here, methods are provided for synthesizing internal standards containing a mixture of fatty acids (up to 10 fatty acids per lipid class). In the methods, the fatty acids in the internal standard mixture (IS mixture) are selected to represent the diversity of chemical structures (lipid molecular species) found in the lipid classes present in the sample type to be analyzed. Table 1 shows the concentration of each fatty acid measured for each corresponding lipid class according to the methods of the present disclosure, and the remaining fatty acids in each lipid class were assigned to the closest internal standard analogue and assigned that measured value; the total concentration for each lipid class was calculated by adding the values (measured and assigned) of all molecular species for that lipid class.

[0056]   In one embodiment, internal standards are provided for each of 10 lipid classes according the composition of fatty acids shown in Table 1. The lipid classes for the internal standards are shown in the first row of Table 1, and the fatty acid "R" groups are listed in column 1 of Table 1. In Table 1, "d" refers to the addition of a deuterium label. For example, 16:0-d9 refers to palmitate with 9 deuterium atoms added

Table 1. Composition of fatty acid mixtures for synthesizing lipid internal standards

	PC	O-PC	LPC	PE	P-PE	LPE	TG	DG	CE	SM
R	16:0-d9/R	O-16:0-d9/R	16:0-d9	18:0-d9/R	P-18:0-d9/R	18:0-d9	16:0-d9/18:1n9/R	16:0-d9/R	CE-d6/R	18:1n9-d9/R
d	-	-	100%	-	-	100%	-	-	-	-
FA14:0	-	-	-	-	-		-	-	-	-
FA16:0	-	-	-	-	-	-	20%	20%	5%	25%
FA18:0	-	-	-	-	-	-	20%	20%	-	-
FA24:0	-	-	-	-	-	-	-	-	-	25%
FA16:1n7	5%	5%	-	-	-	-	-	-	5%	-
FA18:1n9	20%	20%	-	20%	20%	-	20%	20%	20%	25%
FA24:1n9	-	-	-	-	-	-	-	-	-	25%
FA18:2n6	20%	20%	-	20%	20%	-	20%	20%	50%	-
FA18:3n3	5%	5%	-	5%	5%	-	5%	5%	-	-
FA20:3n6	5%	5%	-	5%	5%	-	5%	-	5%	-
FA20:4n6	20%	20%	-	20%	20%	-	5%	5%	5%	-
FA20:5n3	5%	5%	-	5%	5%	-	-	5%	5%	-
FA22:4n6	5%	5%	-	-	-	-	-	-	-	-
FA22:5n3	5%	5%	-	5%	5%	-	-	-	-	-
FA22:6n3	10%	10%	-	20%	20%	-	5%	5%	5%	-
FA17:1n7	-	-	-	-	-	-	-	-	-	-
Sum	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Count	10	10	1	8	8	1	8	8	8	4

[0057]   In one embodiment, exemplary internal standard components are provided for phosphatidylcholine and o-phosphatidylcholine, and the composition of fatty acids at the sn-2 position (sn-2 fatty acids) for these internal standards are displayed in Table 2. The structures of phosphatidylcholine and sn-2 fatty acids comprising the mixture are shown in FIG. 2. The structure of phosphatidylcholine with deuterated palmitate (16:0) in the sn-1 position and a fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 2A. The structure of phosphatidylcholine with deuterated palmitate (16:0) in the sn-1 position and oleate (18:1n9), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 2B. The structures of ten fatty acids (16:1n7, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n3, 22:6n3) in the fatty acid mixture for acylating into the sn-2 position of phosphatidylcholine are shown in FIG. 2C.

Table 2. Composition of sn-2 fatty acids for phosphatidylcholine and o-phosphatidylcholine internal standards

sn2-FA	%	ID	Systematic Name
16:1n7	5%	PC (16:0-d9/16:1n7)	1-(hexadecanoyl-d9)-2-(9Z-hexadecenoyl)-sn-glycero-3-phosphocholine
18:1n9	20%	PC (16:0-d9/18:1n9)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine
18:2n6	20%	PC (16:0-d9/18:2n6)	1-(hexadecanoyl-d9)-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine
18:3n3	5%	PC (16:0-d9/18:3n3)	1-(hexadecanoyl-d9)-2-(9Z,12Z,15Z-octadecatrienoyl)-sn-glycero-3-phosphocholine
20:3n6	5%	PC (16:0-d9/20:3n6)	1-(hexadecanoyl-d9)-2-(8Z,11Z,14Z-eicosatrienoyl)-sn-glycero-3-phosphocholine
20:4n6	20%	PC (16:0-d9/20:4n6)	1-(hexadecanoyl-d9)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine
20:5n3	5%	PC (16:0-d9/20:5n3)	1-(hexadecanoyl-d9)-2-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine
22:4n6	5%	PC (16:0-d9/22:4n6)	1-(hexadecanoyl-d9)-2-(7Z,10Z,13Z,16Z-docosatetraenoyl)-sn-glycero-3-phosphocholine
22:5n3	5%	PC (16:0-d9/22:5n3)	1-(hexadecanoyl-d9)-2-(7Z,10Z,13Z,16Z,19Z-docosapentaenoyl)-sn-glycero-3-phosphocholine
22:6n3	10%	PC (16:0-d9/22:6n3)	1-(hexadecanoyl-d9)-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine

[0058]   In one embodiment, exemplary internal standards are provided for lysophosphatidylcholine internal standards and the structure is shown in FIG. 3.

[0059]   For the phosphatidylethanolamine and p-phosphatidylethanolamine internal standards, the composition of sn-2 fatty acids are listed in Table 3. The structures of phosphatidylethanolamine and sn-2 fatty acids comprising the mixture are shown in FIG. 4. The structure of phosphatidylethanolamine with deuterated stearate (18:0) in the sn-1 position and the fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 4A. The structure of phosphatidylethanolamine with deuterated stearate (18:0) in the sn-1 position and oleate (18:1n9) as an exemplary fatty acid in the sn-2 position is shown in FIG. 4B. The structures of eight fatty acids (18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:5n3, 22:6n3) in the fatty acid mix for acylating into the sn-2 position of phosphatidylethanolamine are shown in FIG. 4C.

Table 3. Composition of sn-2 fatty acids for phosphatidylethanolamine and p-phosphatidylethanolamine internal standards

sn2-FA	%	ID	Systematic Name
18:1n9	20%	PE (18:0-d9/18:1n9)	1-(octadecanoyl-d9)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine
18:2n6	20%	PE (18:0-d9/18:2n6)	1-(octadecanoyl-d9)-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphoethanolamine
18:3n3	5%	PE (18:0-d9/18:3n3)	1-(octadecanoyl-d9)-2-(9Z,12Z,15Z-octadecatrienoyl)-sn-glycero-3-phosphoethanolamine
20:3n6	5%	PE (18:0-d9/20:3n6)	1-(octadecanoyl-d9)-2-(8Z,11Z,14Z-eicosatrienoyl)-sn-glycero-3-phosphoethanolamine
20:4n6	20%	PE (18:0-d9/20:4n6)	1-(octadecanoyl-d9)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphoethanolamine
20:5n3	5%	PE (18:0-d9/20:5n3)	1-(octadecanoyl-d9)-2-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-sn-glycero-3-phosphoethanolamine
22:5n3	5%	PE (18:0-d9/22:5n3)	1-(octadecanoyl-d9)-2-(7Z,10Z,13Z,16Z,19Z-docosapentaenoyl)-sn-glycero-3-phosphoethanolamine
22:6n3	20%	PE (18:0-d9/22:6n3)	1-(octadecanoyl-d9)-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine

[0060]   In one embodiment, exemplary internal standards are provided for lysophosphatidylethanolamine and the structure is shown in FIG. 5.

[0061]   In one embodiment, exemplary internal standards are provided for sphingomyelin and the composition of fatty acids are displayed in Table 4. The structures of sphingomyelin and sn-2 fatty acids comprising the mixture are shown in FIG. 6. The structure of sphingomyelin with deuterium-labeled sphingosine at the first position, and the fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 6A. The structure of sphingomyelin with deuterium-labeled sphingosine at the first position, and palmitate (16:0), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 6B. The structures of four fatty acids (16:0, 18:1n9, 24:0, 24:1n9) in the fatty acid mix for acylating into the sn-2 position of sphingomyelin are shown in FIG. 6C.

Table 4. Composition of fatty acids for sphingomyelin internal standard

sn2-FA	%	ID	Systematic Name
16:0	25%	SM (18:1n9-d9/16:0)	N-(hexadecanoyl)-(sphing-d9)-4-enine-1-phosphocholine
18:1n9	25%	SM (18:1n9-d9/18:1n9)	N-(9Z-octadecenoyl)-(sphing-d9)-4-enine-1-phosphocholine
24:0	25%	SM (18:1n9-d9/24:0)	N-(tetracosanoyl)-(sphing-d9)-4-enine-1-phosphocholine
24:1n9	25%	SM (18:1n9-d9/24:1n9)	N-(15Z-tetracosenoyl)-(sphing-d9)-4-enine-1-phosphocholine

[0062]   In one embodiment, exemplary internal standards are provided for triacylglycerol and the composition of sn-3 fatty acids are listed in Table 5. The structures of triacylglycerol and sn-3 fatty acids comprising the mixture are shown in FIG. 7. The structure of triacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, oleate (18:1n9) at the sn-2 position, and the fatty acid "R" in the sn-3 position is shown in FIG. 7A. The structure of triacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, oleate (18:1n9) at the sn-2 position, and palmitate (16:0) as an exemplary fatty acid in the sn-3 position is shown in FIG. 7B. The structures of eight fatty acids (16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 22:6n3) in the fatty acid mix for labeling the sn-3 position of triacylglycerol are shown in FIG. 7C.

Table 5. Composition of sn-3 fatty acids for triacylglycerol internal standard

sn3-FA	%	ID	Systematic Name
16:0	20%	TG (16:0-d9/18:1n9/16:0)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-hexadecanoyl-sn-glycerol
18:0	20%	TG (16:0-d9/18:1n9/18:0)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-octadecanoyl-sn-glycerol
18:1n9	20%	TG (16:0-d9/18:1n9/18:1n9)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(9Z-octadecenoyl)-sn-glycerol
18:2n6	20%	TG (16:0-d9/18:1n9/18:2n6)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(9Z,12Z-octadecadienoyl)-sn-glycerol
18:3n3	5%	TG (16:0-d9/18:1n9/18:3n3)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(9Z,12Z,15Z-octadecatrienoyl)-sn-glycerol
20:3n6	5%	TG (16:0-d9/18:1n9/20:3n6)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(8Z,11Z,14Z-eicosatrienoyl)-sn-glycerol
20:4n6	5%	TG (16:0-d9/18:1n9/20:4n6)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycerol
22:6n3	5%	TG (16:0-d9/18:1n9/22:6n3)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-3-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycerol

[0063]   In one embodiment, exemplary internal standards are provided for diacylglycerol and the composition of sn-2 fatty acids are listed in Table 6. The structures of diacylglycerol and sn-2 fatty acids comprising the mixture are shown in FIG. 8. The structure of diacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, and a fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 8A. The structure of diacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, and palmitate (16:0), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 8B. The structures of eight fatty acids (16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, 22:6n3) in the fatty acid mix for labeling the sn-2 position of diacylglycerol are shown in FIG. 8C.

Table 6. Composition of sn-2 fatty acids for diacylglycerol internal standard

sn2-FA	%	ID	Systematic Name
16:0	20%	DG (16:0-d9/16:0)	1-(hexadecanoyl-d9)-2-hexadecanoyl-sn-glycerol
18:0	20%	DG (16:0-d9/18:0)	1-(hexadecanoyl-d9)-2-octadecanoyl-sn-glycerol
18:1n9	20%	DG (16:0-d9/18:1n9)	1-(hexadecanoyl-d9)-2-(9Z-octadecenoyl)-sn-glycerol
18:2n6	20%	DG (16:0-d9/18:2n6)	1-(hexadecanoyl-d9)-2-(9Z,12Z-octadecadienoyl)-sn-glycerol
18:3n3	5%	DG (16:0-d9/18:3n3)	1-(hexadecanoyl-d9)-2-(9Z,12Z,15Z-octadecatrienoyl)-sn-glycerol
20:4n6	5%	DG (16:0-d9/20:4n6)	1-(hexadecanoyl-d9)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycerol
20:5n3	5%	DG (16:0-d9/20:5n3)	1-(hexadecanoyl-d9)-2-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-sn-glycerol
22:6n3	5%	DG (16:0-d9/22:6n3)	1-(hexadecanoyl-d9)-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycerol

[0064]   In one embodiment, exemplary internal standards are provided for cholesteryl ester and the composition of fatty acids is listed in Table 7. The structures of cholesteryl ester and fatty acids comprising the mixture are shown in FIG. 9. The structure of cholesteryl ester with deuterium labels at the n6 position, and the fatty acid "R" acylated to the hydroxyl group is shown in FIG. 9A. The structure of cholesteryl ester with deuterium labels at the n6 position, and linoleate (18:2n6), as the exemplary fatty acid, acylated to the hydroxyl group is shown in FIG. 9B. The structure of eight fatty acids (16:0, 16:1n7, 18:1n9, 18:2n6, 20:3n6, 20:4n6, 20:5n3, 22:6n3) in the fatty acid mix for use in acylating the hydroxyl group of cholesteryl ester are shown in FIG. 9C.

Table 7. Composition of fatty acids for cholesteryl ester internal standard

Fatty Acid	%	ID	Systematic Name
16:0	5%	CE-d6/16:0	cholest-5-en-3B-yl-d6 hexadecanoate
16:1n7	5%	CE-d6/16:1n7	cholest-5-en-3B-yl-d6 (9Z-hexadecenoate)
18:1n9	20%	CE-d6/18:1n9	cholest-5-en-3B-yl-d6 (9Z-octadecenoate)
18:2n6	50%	CE-d6/18:2n6	cholest-5-en-3B-yl-d6 (9Z,12Z-octadecadienoate)
20:3n6	5%	CE-d6/20:3n6	cholest-5-en-3B-yl-d6 (8Z,11Z,14Z-eicosatrienoate)
20:4n6	5%	CE-d6/20:4n6	cholest-5-en-3B-yl-d6 (5Z,8Z,11Z,14Z-eicosatetraenoate)
20:5n3	5%	CE-d6/20:5n3	cholest-5-en-3B-yl-d6 (5Z,8Z,11Z,14Z,17Z-eicosapentaenoate)
22:6n3	5%	CE-d6/22:6n3	cholest-5-en-3B-yl-d6 (4Z,7Z,10Z,13Z,16Z, 19Z-docosahexaenoate)

[0065]   In one embodiment, exemplary internal standards are provided for free fatty acids and the composition of fatty acids are listed in Table 8. The structures of the free fatty acids comprising the mixture are shown in FIG. 10. The structure of the free fatty acid palmitate (16:0) with deuterium labels is shown in FIG. 10A. The structure of the free fatty acid, 17:1n7, is shown in FIG. 10B.

Table 8. Composition of free fatty acid internal standard

%	ID	Systematic Name
50%	FA 16:0-d9	Hexadonic-d9 acid
50%	FA 17:1n7	10Z-heptadecenoic acid

Methods for generating lipid internal standards

[0066]   In one embodiment of the present disclosure a method is provided for synthesizing one or more mixtures of lipid molecules for use as an internal standard representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising one or more of: attaching a mixture of at least two different isotopically-labeled fatty acids to a lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; or attaching a single

isotopically-labeled fatty acid to a lipid backbone at a first position through an acylation reaction for a lipid class having at least two fatty acids and attaching a mixture of at least two different fatty acids to a separate position on the lipid backbone through an acylation reaction, wherein the mixture of the at least two different fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest. The corresponding lipid class can include one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

[0067]   In one embodiment of the present disclosure a method is provided for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising: attaching an isotopically-labeled fatty acid at a first position on a lipid backbone through an acylation reaction for a lipid class having at least two acyl groups; and attaching a mixture of at least two different fatty acids to the lipid backbone at a separate position through an acylation reaction, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecules present in the corresponding lipid class in the sample of interest. In the

method, the one or more lipid classes having at least two acyl groups can include triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6.

[0068]   In one embodiment of the present disclosure, a method is provided for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising: attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecules present in the one corresponding lipid class in the sample of interest. In the method, the one or more lipid classes having at least one acyl group can comprise triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7. The one or more mixtures of fatty acids

in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

[0069]   In the methods provided above, the sample of interest can be a complex mixture comprised of lipid molecules, a biological sample such as a plant sample or an animal sample. The animal sample may be from a mammal such as, for example, a human, a mouse, a non-human primate, a rabbit or other mammal, or a non-mammal sample such as, for example, a zebra fish sample. The biological sample of interest can include blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, or a skin sample.

[0070]   In the methods provided above, the methods can further include performing quantitative fatty acid analysis to quantify the amount of each of the fatty acids in the mixture.

[0071]   In the methods provided above, the fatty acid can be a saturated fatty acid.

[0072]   In the methods provided above, the isotopic label can include any isotopic label including ^{2}H, ^{13}C, or ^{15}N.

[0073]   In one embodiment of the methods above, the first position on the lipid backbone can be a sn-1 position. The first position on the lipid backbone can be a sn-1 position and the separate position on the lipid backbone can be a sn-2 or a sn-3 position. In an alternate embodiment, the first position on the lipid backbone can be a sn-2 position. The first position can be a sn-2 and the separate position can be a sn-1 or sn-3 position. In another embodiment, the first position on the lipid backbone can be a sn-3 position. The first position can be a sn-3 position and the separate position can be a sn-1 or sn-2 position.

[0074]   In the methods provided above, the one or more lipid classes having at least two acyl groups can consist of phosphatidylcholines or o-phosphatidylcholines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or wherein the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled hexadecanoyl-d9 (16:0-d9) can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6),

7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the sn-2 position can be present at the ratios shown in Table 2.

[0075]   In the methods provided above, the one or more lipid classes having at least two acyl groups can consist of phosphatidylethanolamines and p-phosphatidylethanolamines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or wherein the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled octadecanoyl-d9 (18:0-d9) can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 3.

[0076]   In the methods provided above, the one or more lipid classes having at least two acyl groups can consist of sphingomyelins, wherein deuterium-labeled sphingosine can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 4.

[0077]   In the methods provided above, the one or more lipid classes having at least two acyl groups can consist of diacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or wherein the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 6.

[0078]   In the methods provided above, the one or more lipid classes having at least two acyl groups can consist of triacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 or sn-3 position, or wherein the first position can be an sn-2 position and the separate position can be an sn-1 or sn-3 position, or wherein the first position can be a sn-3 position and the separate position can be a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, wherein oleate (18:1n9) can be present at one separate position, and wherein the mixture of fatty acids at another separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 5.

[0079]   In the methods provided above, the one or more lipid classes having at least one acyl group can consist of cholesteryl esters, wherein the single position can be a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group can comprise hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3). The mixture of fatty acids at the hydroxyl group can be present at the ratios shown in Table 7.

Compositions for use as lipid internal standards

[0080]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of: a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or a lipid backbone having a single isotopically-labeled fatty acid at a first

position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids, wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest. The lipid class can include one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

[0081]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups; and a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest. In the composition, the lipid class can include one or more of

triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6.

[0082]   In one embodiment of the present disclosure, a composition is provided for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having one or more isotopic labels, wherein the lipid backbone is for a lipid class having at least one acyl group; and a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest. In the composition, the lipid class can include one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7. The one or more mixtures of fatty acids in each of the one or more

mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

[0083]   In the compositions provided above, the sample of interest can be a complex mixture comprised of lipid molecules, a biological sample such as a plant sample or an animal sample. The animal sample may be from a mammal such as, for example, a human, a mouse, a non-human primate, a rabbit or other mammal, or a non-mammal such as, for example, a zebra fish sample. The biological sample of interest can include blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, or a skin sample.

[0084]   In the compositions provided above, the isotopic label can include any isotopic label including ^{2}H, ^{13}C, or ^{15}N.

[0085]   In the compositions provided above, the fatty acid can be a saturated fatty acid.

[0086]   In the compositions provided above, the first position on the lipid backbone can be a sn-1 position. The first position on the lipid backbone can be a sn-1 position and the separate position on the lipid backbone can be a sn-2 or a sn-3 position. In an alternate embodiment, the first position on the lipid backbone can be a sn-2 position. The first position can be a sn-2 and the separate position can be a sn-1 or sn-3 position. In another embodiment, the first position on the lipid backbone can be a sn-3 position. The first position can be a sn-3 position and the separate position can be a sn-1 or sn-2 position.

[0087]   In the compositions provided above, one of the lipid classes can consist of phosphatidylcholines or o-phosphatidylcholines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled hexadecanoyl-d9 (16:0-d9) can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 2.

[0088]   In the compositions provided above, one of the lipid classes can consist of phosphatidylethanolamines and p-phosphatidylethanolamines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled octadecanoyl-d9 (18:0-d9) can be present at the first position , and wherein the mixture of fatty acids at the separate position can comprise 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 3.

[0089]   In the compositions provided above, one of the lipid classes can consist of sphingomyelins, wherein deuterium-labeled sphingosine can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 4.

[0090]   In the compositions provided above, one of the lipid classes can consist of diacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 6.

[0091]   In the compositions provided above, one of the lipid classes can consist of triacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 or sn-3 position, or the first position can be a sn-2 position and the separate position can be a sn-1 or sn-3 position, or the first position can be a sn-3 position and the separate position can be a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, wherein oleate (18:1n9) can be present at a sn-2 position, and wherein the

mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acids at the separate position can be present at the ratios shown in Table 5.

[0092] In the compositions provided above, one of the lipid classes can consist of cholesteryl esters, wherein the single position can be a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group can comprise hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3). The mixture of fatty acids at the hydroxyl group can be present at the ratios shown in Table 7.

Methods for detection and quantification of lipid molecules in samples of interest

[0093] In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, the method comprising: i) adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, the composition comprising one or more of: a) a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; b) an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or c) a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids, wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and ii) one of detecting and quantifying

the lipid molecular species present in each of the one or more corresponding lipid classes in the sample of interest by using the composition having the one or more representative mixtures of lipid molecules as an internal standard.

[0094]   In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, comprising: adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, wherein each mixture of lipid molecules comprises i) a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups; and ii) a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest; and one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest by using the representative mixture of lipid molecules as an internal standard.

[0095]   In one embodiment of the present disclosure, a method is provided for one of detecting and quantifying lipid molecules present in a sample of interest, comprising: adding to a sample of interest a known amount of a composition having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest, wherein each mixture of lipid molecules comprises i) a lipid backbone having one or more isotopic labels, wherein the lipid backbone is for a lipid class having at least one acyl group; and ii) a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and one of detecting and quantifying the lipid

molecular species present in the corresponding lipid class in the sample of interest by using the representative mixture of lipid molecules as an internal standard.

[0096]   The method of detecting and/or quantifying the lipid molecular species present in the sample of interest can include one or a combination of mass spectrometry (MS), high performance liquid chromatography (HPLC), isocratic HPLC, gradient HPLC, normal phase chromatography, reverse phase HPLC, size exclusion chromatography, ion exchange chromatography, capillary electrophoresis, microfluidics, chromatography, gas chromatography (GC), thin-layer chromatography (TLC), and combinations thereof. The method for detecting and/or quantifying the lipid molecular species present in the biological sample can include the use of mass spectrometry (MS).

[0097]   In the method of detecting and/or quantifying the lipid molecular species present in the sample of interest, the sample of interest can be a complex mixture comprised of lipid molecules, a biological sample such as a plant sample or an animal sample. The animal sample may be from a mammal such as, for example, a human, a mouse, a non-human primate, a rabbit or other mammal, or a non-mammal such as, for example, a zebra fish sample. The biological sample of interest can include blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, or a skin sample.

[0098]   In the method of detecting and/or quantifying the lipid molecular species present in the sample of interest, the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7. The one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

Kit

[0099]   Generally, the kits of the present disclosure include one or more internal standards and instructions for using the internal standards to detect and/or quantify lipids in the sample of interest. The kit may further include one or more control samples and sample collection receptacles. The internal standard compositions within a kit can be packaged together in various combinations of one or more lipid classes or can be packaged in separate vials or containers. A

kit may include labels and/or packaging inserts setting out instructions for preparation and use, specimen collection receptacles, a transportation container, and/or a mailer for shipping. Additional kit components in separate packaging could include buffers and other reagents for the detection and/or quantification of lipids in a sample of interest.

[00100]   The kit can comprise one or more internal standard mixtures. In one embodiment, the kit can contain an internal standard mixture comprising isotopically labeled fatty acids wherein at least one isotopically labeled fatty acid is present from each lipid class in the sample of interest. In one embodiment, the kit may contain a set of internal standards comprising the lipid classes and isotopically labeled fatty acids presented in Table 1. In a further embodiment, the isotopically labeled fatty acids in the set of internal standards are present at the ratios given in Table 1.

[00101]   The kit may further comprise a volumetric container. The volumetric container may be any container (i.e., a cup, vial, microfuge tube, microtiter plate etc.) suitable for holding a liquid sample. The volumetric container may optionally contain volumetric measurements which may be useful in measuring out a desirable amount of the sample or other reagents. The volumetric container may be made of any material (e.g., plastics, aluminum, stainless steel). The internal volume of the volumetric container depends on the type of sample to be collected. The volumetric container can include a body and a cap. In some embodiments, the internal standard material may be attached to the cap. In some embodiments, the internal standard material may be coated on the internal volume of the body of the volumetric container.

[00102]   In some embodiments, the volumetric container may additionally be configured for the type of sample collection contemplated and used for collection of the specimen. In other aspects, a specimen collection receptacle is separately provided in the kit, and may be in the form of a cup, vial, microfuge tube.

[00103]   The kit may optionally comprise a transportation container. The transportation container may be any structure suitable for transportation of samples. The container is configured such that the sample material can be packed into the container and the container may be sealed.

[00104]   The kit may optionally include an extraction solution.

[00105]   In one embodiment of the present disclosure, a kit is provided comprising: i) one or more mixtures of lipid molecules for use as an internal standard, wherein each of the one or more mixtures of lipid molecules is representative of the composition of lipid molecular species

present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of: a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups, wherein the mixture of at least two different fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and ii) instructions for using the one or more mixtures of lipid molecules as the internal standard for one of detecting and quantifying the lipid molecular species present in the lipid class in the sample of interest. The lipid class can include one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

[00106]   In one embodiment of the present disclosure, a kit is provided comprising: i) one or more mixtures of lipid molecules for use as an internal standard, wherein each of the one or more mixtures of lipid molecules is representative of the composition of lipid molecular species present in each of one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising: a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone and a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at

least two acyl groups, or a lipid backbone having one or more isotopic labels and a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group, wherein the mixture of fatty acids is representative of the fatty acids that occur in the lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and ii) instructions for using the one or more mixtures of lipid molecules as the internal standard for one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest. The lipid class having at least two acyl groups can include one or more of triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters. The lipid class having at least one acyl group can include one or more of triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax diesters cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, wax esters, 1-monoacylglycerol.

[00107]   The kits can include two or more of the mixtures of the lipid molecules representative of the composition of lipid molecular species present in two or more of the corresponding lipid classes in the sample of interest. The two or more mixtures of the lipid molecules can be packaged as a single component. The two or more mixtures of the lipid molecules can be packaged as separate components.

[00108]   The kits may further include reagents for the detecting and quantifying.

[00109]   The kits may further include a control sample having a known concentration of the composition of the lipid molecular species.

[00110]   The kits may further include a mixture of free fatty acids for use as an internal standard that is representative of the composition of free fatty acids in the sample of interest. The free fatty acids for use in the kit may comprise the free fatty acids shown in Table 8.

[00111]   In the kits, the sample of interest can be a complex mixture comprised of lipid molecules, a biological sample such as a plant sample or an animal sample. The animal sample may be from a mammal such as, for example, a human, a mouse, a non-human primate, a rabbit or other mammal, or a non-mammal such as, for example, a zebra fish sample. The biological sample of interest can include blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, or a skin sample.

[00112]   In the kits, the fatty acid can be a saturated fatty acid.

[00113]   In the kits, the isotopic label can include ^{2}H, ^{13}C, or ^{15}N.

[00114]   In the kits, the mixtures of fatty acids in each of the one or more mixtures of lipid molecules can include the mixtures of lipid molecular species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, or Table 8. In the kits, the mixtures of fatty acids in each of the one or more mixtures of lipid molecules can consist of one or more of the mixtures of lipid molecular species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, or Table 8.

[00115]   In the kit, the first position on the lipid backbone can be a sn-1 position. The first position on the lipid backbone can be a sn-1 position and the separate position on the lipid backbone can be a sn-2 or a sn-3 position.

[00116]   In the kit, one of the lipid classes can consist of phosphatidylcholines or o-phosphatidylcholines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled hexadecanoyl-d9 (16:0-d9) can be present at the first position, and wherein the mixture of fatty acid groups at the separate position can comprise 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acid groups at the separate position can be present at the ratios shown in Table 2.

[00117]   In the kit, one of the lipid classes can consist of phosphatidylethanolamines and p-phosphatidylethanolamines, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein a deuterium-labeled octadecanoyl-d9 (18:0-d9) can be present at the first position , and wherein the mixture of fatty acid groups at the separate position can comprise 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acid groups at the separate position can be present at the ratios shown in Table 3.

[00118]   In the kit, one of the lipid classes can consist of sphingomyelins, wherein deuterium-labeled sphingosine can be present at the first position, and wherein the mixture of fatty acids at the separate position can comprise hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9). The mixture of fatty acid groups at the separate position can be present at the ratios shown in Table 4.

[00119]   In the kit, one of the lipid classes can consist of diacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 position, or the first position can be a sn-2 position and the separate position can be a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, and wherein the mixture of fatty acid groups at the separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acid groups at the separate position can be present at the ratios shown in Table 6.

[00120]   In the kit, one of the lipid classes can consist of triacylglycerols, wherein the first position can be a sn-1 position and the separate position can be a sn-2 or sn-3 position, or the first position can be a sn-2 position and the separate position can be a sn-1 or sn-3 position, or the first position can be a sn-3 position and the separate position can be a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) can be present at the first position, wherein oleate (18:1n9) can be present at a one separate position, and wherein the mixture of fatty acid groups at another separate position can comprise hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-

octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl

(18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and

4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3). The mixture of fatty acid groups at the

separate position can be present at the ratios shown in Table 5.

[00121]   In the kit, one of the lipid classes can comprise cholesteryl esters,

lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine,

lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-

diacylglycerol, wax esters, or 1-monoacylglycerol.

[00122]   In the kit, one of the lipid classes can consist of cholesteryl esters, wherein the single

position is a hydroxyl group, and wherein the mixture of acyl groups attached to the hydroxyl

group comprises hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoate (18:1n9),

9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-

eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and

4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3). The mixture of acyl groups at the

hydroxyl group can be present at the ratios shown in Table 7.

Methods of Measurement of Lipid Molecules

[00123]   Assays for lipid metabolite or lipid molecule content may be performed on any

sample type including, for example, a body fluid sample (e.g., blood, blood plasma, urine,

cerebral spinal fluid (CSF)), a tissue sample, a cellular sample, a skin sample, a solution, a

complex mixture, in vitro cultured cells, and cell culture media. In some embodiments, the

amounts of the lipid molecules are determined from sample(s) selected from the group consisting

of complex mixtures of lipids, blood, plasma, serum, isolated lipoprotein fraction, saliva, urine,

lymph fluid, and cerebrospinal fluid. In some embodiments, the assays may be performed on

whole blood, plasma, serum, or isolated lipoprotein fractions. In some embodiments, the

sample(s) are plasma or serum.

[00124]   In some embodiments, multiple different lipid molecules are measured in the same

sample. In other embodiments, each of multiple lipid molecules is measured from a different

sample. If multiple samples are used, the samples may be from the same or different body fluids

of the subject.

[00125]   The lipid molecules and other biomarkers may readily be isolated and/or quantified

by methods known to those of skill in the art, including, but not limited to, methods utilizing:

mass spectrometry (MS), high performance liquid chromatography (HPLC), isocratic HPLC, gradient HPLC, normal phase chromatography, reverse phase HPLC, size exclusion chromatography, ion exchange chromatography, capillary electrophoresis, microfluidics, chromatography, gas chromatography (GC), thin-layer chromatography (TLC), immobilized metal ion affinity chromatography (IMAC), affinity chromatography, immunoassays, and/or colorimetric assays. In some embodiments, the methods of the invention utilize MS to determine lipid molecule content.

[00126]   For fatty acid methyl ester (FAME) analysis, lipids were extracted by the method of Folch et al. (*J Biol Chem* **226**:497-509) using chloroform:methanol (2:1 v/v). Neutral lipids were separated from polar lipids by solid phase chromatography. The polar lipid fraction was separated into individual lipid classes using the Agilent Technologies 1100 Series LC, and the lipid class fractions were collected for fatty acid analysis. Neutral lipids were separated into individual lipid classes by thin-layer chromatography using a solvent system consisting of petroleum ether/diethyl ether/acetic acid (80:20:1), and the lipid class fractions were collected for fatty acid analysis. Each lipid class was transesterified in 1% sulfuric acid in methanol in a sealed vial under a nitrogen atmosphere at 100°C for 45 minutes. The resulting fatty acid methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene and prepared for GC analysis by sealing the hexane extracts under nitrogen. Fatty acid methyl esters were separated and quantified by capillary GC (Agilent Technologies 6890 Series GC) equipped with a 30 m DB 88 capillary column (Agilent Technologies) and a flame ionization detector.

[00127]   Various analytical methods are well known to those of skill in the art, and are further described in the following documents, which are herein incorporated by reference in their entirety: MS: Cyr D, et al. A GCIMS validated method for the nanomolar range determination of succinylacetone in amniotic fluid and plasma: an analytical tool for tyrosinemia type I. J Chromatogr B Analyt Techno) Biomed Life Sci. 2006 Feb 17;832(1):24- 9; Vogeser M. Abstract Liquid chromatography-tandem mass spectrometry--application in the clinical laboratory. Clin Chern Lab Med. 2003 Feb;41(2):117-26. HPLC: Khalil PN, et al. Validation and application of a high-performance liquid chromatographic-based assay for determination of the inosine 5'-monophosphate dehydrogenase activity in erythrocytes. J Chromatogr B Analyt Techno) Biomed Life Sci. 2006 May 23; Fouassier M, et al. Determination of serotonin release from platelets by

HPLC and ELISA in the diagnosis of heparin-induced thrombocytopenia: comparison with reference method by [C)-serotonin release assay; J Thromb Haemost. 2006 May;4(5): 1136-9; Badiou S, et al. Determination of plasma amino acids by fluorescent derivatization and reversed-phase liquid chromatographic separation. Clin Lab. 2004;50(3-4): 153-8; Brunelli T, et al. Comparison of three methods for total homocysteine plasma determination. Clin Lab. 200 I ;47(7-8):393-7. CE: Zinellu A, et al. Assay for the simultaneous determination of guanidinoacetic acid, creatinine and creatine in plasma and urine by capillary electrophoresis UV -detection. J Sep Sci. 2006 Mar;29(5):704-8; Jabeen R, et al. Capillary electrophoresis and the clinical laboratory. Electrophoresis. 2006 May 23; Gao P, et al. Rapid detection of Staphylococcus aureus by a combination of monoclonal antibody-coated latex and capillary electrophoresis. Electrophoresis. 2006 May;27(9):1784-9. Microfluidics: Johannessen EA, et al. A suspended membrane nanocalorimeter for ultralow volume bioanalysis. IEEE Trans Nanobioscience. 2002 Mar;l(l):29-36; Herrmann M, et al. Enzymatically-generated fluorescent detection in micro-channels with internal magnetic mixing for the development of parallel microfluidic ELISA; Lab Chip. 2006 Apr;6(4):555-60. Epub 2006 Mar 3; Yang S, et al. Blood plasma separation in microfluidic channels using flow rate control. ASAIO J. 2005 Sep-Oct;51 (5):585-90; Dupuy AM, et al. Protein biochip systems for the clinical laboratory; Clin Chern Lab Med. 2005;43{12):1291-302. Chromatography: Paterson S, et al. Validation of techniques to detect illicit heroin use in patients prescribed pharmaceutical heroin for the management of opioid dependence. Addiction. 2005 Dec; 1 00(12): 1832-9; Bottcher M, et al. Evaluation of buprenorphine CEDIA assay versus GC-MS and ELISA using urine samples from patients in substitution treatment. J Anal Toxicol. 2005 Nov-Dec;29(8):769-76; Julak J. Chromatographic analysis in bacteriologic diagnostics of blood cultures, exudates, and bronchoalveolar lavages. Prague Med Rep. 2005; 1 06(2): 175-94; Boettcher M, et al. Precision and comparability of Abuscreen OnLine assays for drugs of abuse screening in urine on Hitachi 917 with other immunochemical tests and with GC/MS. Clin Lab. 2000;46(1-2):49-52. Immunoassays: Boettcher M, et al. Precision and comparability of Abuscreen OnLine assays for drugs of abuse screening in urine on Hitachi 917 with other immunochemical tests and with GC/MS. Clin Lab. 2000;46(1-2):49-52; Westermann J, et al. Simple, rapid and sensitive determination of epinephrine and norepinephrine in urine and plasma by non-competitive enzyme immunoassay, compared with HPLC method. Clin Lab. 2002;48(1-2):61-71; Aoyagi K, et al. Performance of a

conventional enzyme immunoassay for hepatitis C virus core antigen in the early phases of hepatitis C infection. Clin Lab. 200 I ;47(3-4): 119-27; Hub I W, et al. A multi-center quality control study of different CA 15-3 immunoassays. Clin Lab. 2005;51(11-12):641-5; Haller CA, et al. Comparison of an automated and point-of-care immunoassay to GC-MS for urine oxycodone testing in the clinical laboratory. J Anal Toxicol. 2006 Mar;30(2): 106-11; Bayer M, et al. Evaluation of a new enzyme-linked immunosorbent assay for the determination of neopterin. Clin Lab. 2005 ;51 (9-1 0):495-504; Groche D, et al. Standardization of two immunological HbA 1 c routine assays according to the new IFCC reference method. Clin Lab. 2003;49(11-12):657-6 1; Ivan 0, et al; German KIMS Board. Applicability of recently established reference values for serum insulin-like growth factor I: A comparison of two assays-- an (automated) chemiluminescence immunoassay and an enzyme-linked immunosorbent assay. Clin Lab. 2005;51(7-8):381-7. Colormetric assays: Kramer KA, et al. Automated spectrophotometric analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts. Clin Chem. 2005 Nov;51(11):2110-6; Groche D, et al. Standardization of two immunological HbA 1 c routine assays according to the new IFCC reference method. Clin Lab. 2003;49(11-12):657-61; WolfPL. History of diagnostic enzymology: A review of significant investigations. Clin Chim Acta. 2006 Mar 24.

[00128] The TRUEMASS analytical platform may also be used for the methods of the invention. TRUEMASS is an analytical platform that may be used to get quantitative data from a sample on approximately 400 individual metabolites involved in structural and energetic lipid metabolism such as triglyceride, cholesterol ester and phospholipid metabolism. This platform is useful in profiling diseases as structural and energetic lipids are central components of metabolism and integrated into virtually every biological process in the body. A data set for a sample, for example a plasma, serum or other biological sample, comprises the quantitative measurement of free cholesterol and the following fatty acids from phosphatidylcholines, phosphatidylethanolamines, lyso-phosphatidylcholines, triglycerides, diglycerides, free fatty acids, and cholesterol esters: 14:0, 15:0, 16:0, 18:0,20:0,22:0,24:0, 14:1n5, l6:1n7, t16:1n7,18:1n9, t18:1n9, 18:ln7, 18:2n6, t18:2n6, 18:3n6, 18:3n3, 18:4n3, 20:ln9, 20:2n6, 20:3n9, 20:3n6, 20:4n6, 20:3n3, 20:4n3, 20:5n3, 22:1 n9, 22:2n6, 22:4n6, 22:5n3, 22:6n3, 24:1 n9, 24:6n3 and plasmalogen derivatives of 16:0, 18:0, 18: 1n9 and 18:1 n7. Methods for using TRUEMASS are known to those of skill in the art, and are also described in the following

documents, which are herein incorporated by reference in their entirety: U.S. Patent Application No. 11/296,829 (filed 12/6/05); Mutch DM, et al. An integrative metabolism approach identifies stearoyi-CoA desaturase as a target for an arachidonate-enriched diet. FASEB J. 2005 Apr;19(6):599-601. Epub 2005 Jan 24; Stone SJ, et al. Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. J Bioi Chern. 2004 Mar 19;279(12):11767-76; Watkins SM, et al. Phosphatidylethanolamine-N-methyltransferase activity and dietary choline regulate liver-plasma lipid flux and essential fatty acid metabolism in mice.J Nutr. 2003 Nov; 133(11):3386-91; Watkins SM, et al. Lipid metabolome-wide effects of the PPARgamma agonist rosiglitazone. Lipid Res. 2002 Nov;43(11):1809-17.

[00129]   The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1. Synthetic Composition of Internal Standard Mixtures.

[00130]   In contrast to traditional lipidomic strategies which typically use only a single internal standard per broad lipid class (e.g., phospholipids), here, internal standards containing a mixture of fatty acids (up to 10 fatty acids per lipid class) were synthesized. The fatty acids in the internal standard mixture (IS mixture) were selected to represent the diversity of chemical structures (lipid molecular species) found in the lipid classes present in the sample type to be analyzed. The concentration of each fatty acid presented in Table 1 was measured for each lipid class, and all remaining fatty acids in the lipid class were assigned to the closest internal standard analogue and assigned that measured value; the total concentration for each lipid class was calculated by adding the values (measured and assigned) of all molecular species for that lipid class.

[00131]   Internal standards for each of 10 lipid classes were synthesized according the composition of fatty acids shown in Table 1. The lipid classes for internal standards are shown in the first row of Table 1, and the fatty acid "R" groups are listed in column 1 of Table 1. In Table 1, "d" refers to the addition of a deuterium label. For example, 16:0-d9 refers to palmitate with 9 deuterium atoms added.

Example 2. Synthesis and Description of Internal Standards and Mixtures Thereof

[00132]   Exemplary phospholipid internal standards for phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, and p-phosphatidylethanolamine were synthesized starting with a glycerol phosphate head group and acylating deuterium-labeled palmitate (16:0) in the sn-1 position; and acylating a mixture of unsaturated fatty acids in the sn-2 position. The labeling strategy for the phospholipids, phosphatidylcholine and phosphatidylethanolamine, is represented in FIG. 1.

[00133]   For the exemplary internal standard components phosphatidylcholine and o-phosphatidylcholine, the composition of fatty acids at the sn-2 position (sn-2 fatty acids) are displayed in Table 2. The structures of phosphatidylcholine and sn-2 fatty acids comprising the mixture are shown in FIG. 2. The structure of phosphatidylcholine with deuterated palmitate (16:0) in the sn-1 position and a fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 2A. The structure of phosphatidylcholine with deuterated palmitate (16:0) in the sn-1 position and oleate (18:1n9), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 2B. The structures of ten fatty acids (16:1n7, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n3, 22:6n3) in the fatty acid mixture for acylating into the sn-2 position of phosphatidylcholine are shown in FIG. 2C.

[00134]   Exemplary lysophosphatidylcholine internal standards were synthesized starting with the phospholipid head group and acylating deuterium-labeled palmitate (16:0). The structure is shown in FIG. 3.

[00135]   For phosphatidylethanolamine and p-phosphatidylethanolamine internal standards, the composition of sn-2 fatty acids are listed in Table 3. The structures of phosphatidylethanolamine and sn-2 fatty acids comprising the mixture are shown in FIG. 4. FIG.The structure of phosphatidylethanolamine with deuterated stearate (18:0) in the sn-1 position and the fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 4A. The structure of phosphatidylethanolamine with deuterated stearate (18:0) in the sn-1 position and oleate (18:1n9) as an exemplary fatty acid in the sn-2 position is shown in FIG. 4B. The structures of eight fatty acids (18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:5n3, 22:6n3) in the fatty acid mix for acylating into the sn-2 position of phosphatidylethanolamine are shown in FIG. 4C.

[00136] Lysophosphatidylethanolamine internal standards were synthesized starting with the phospholipid head group and acylating deuterium-labeled stearate (18:0). The structure is shown in FIG. 5.

[00137] Sphingomyelin internal standards were synthesized starting with a phosphocholine head group, acylating a deuterium-labeled sphingosine to the sn-1 position, and acylating a mixture of fatty acids into the sn-2 position. The acylated mixture of fatty acids in the sn-2 position may comprise, for example, 16:0, 18:1n9, 24:0, and 24:1n9.

[00138] For the exemplary internal standard component, sphingomyelin, the composition of fatty acids are displayed in Table 4. The structures of sphingomyelin and sn-2 fatty acids comprising the mixture are shown in FIG. 6. FIG.The structure of sphingomyelin with deuterium-labeled sphingosine at the first position, and the fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 6A. The structure of sphingomyelin with deuterium-labeled sphingosine at the first position, and palmitate (16:0), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 6B. The structures of four fatty acids (16:0, 18:1n9, 24:0, 24:1n9) in the fatty acid mix for acylating into the sn-2 position of sphingomyelin are shown in FIG. 6C.

[00139] Triacylglycerol internal standards were synthesized starting with a diacylglycerol backbone, acylating deuterium-labeled palmitate (16:0) at the sn-1 position, acylating oleate (18:1n9) at the sn-2 position and acylating a mixture of fatty acids into the sn-3 position. The acylated mixture of fatty acids at the sn-3 position may comprise, for example, 16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6 and 22:6n3.

[00140] For the exemplary internal standard component, triacylglycerol, the composition of sn-3 fatty acids are listed in Table 5. The structures of triacylglycerol and sn-3 fatty acids comprising the mixture are shown in FIG. 7. FIG.The structure of triacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, oleate (18:1n9) at the sn-2 position, and the fatty acid "R" in the sn-3 position is shown in FIG. 7A. The structure of triacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, oleate (18:1n9) at the sn-2 position, and palmitate (16:0) as an exemplary fatty acid in the sn-3 position is shown in FIG. 7B. The structures of eight fatty acids (16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:3n6, 20:4n6, 22:6n3) in the fatty acid mix for labeling the sn-3 position of triacylglycerol are shown in FIG. 7C.

[00141] Exemplary diacylglycerol internal standards were synthesized starting with a glycerol backbone, acylating deuterium-labeled palmitate (16:0) at the sn-1 position, and acylating a

mixture of fatty acids into the sn-2 position. The acylated mixture of fatty acids at the sn-2 position may comprise, for example, 16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, and 22:6n3.

[00142] For example, for diacylglycerol, the composition of sn-2 fatty acids are listed in Table 6. The structures of diacylglycerol and sn-2 fatty acids comprising the mixture are shown in FIG. 8. The structure of diacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, and a fatty acid, denoted by "R", in the sn-2 position is shown in FIG. 8A. The structure of diacylglycerol with deuterium-labeled palmitate (16:0) at the sn-1 position, and palmitate (16:0), as an exemplary fatty acid, in the sn-2 position is shown in FIG. 8B. The structures of eight fatty acids (16:0, 18:0, 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, 22:6n3) in the fatty acid mix for labeling the sn-2 position of diacylglycerol are shown in FIG. 8C.

[00143] Exemplary cholesteryl ester internal standards were synthesized starting with a cholesterol backbone, adding deuterium labels in the n6 position, and acylating a mixture of fatty acids to the hydroxyl group. The acylated mixture of fatty acids acylated to the hydroxyl group may comprise, for example, 16:0, 16:1n7, 18:1n9, 18:2n6, 20:3n6, 20:4n6, 20:5n3, and 22:6n3.

[00144] For example, for cholesteryl ester, the composition of fatty acids is listed in Table 7. The structures of cholesteryl ester and fatty acids comprising the mixture are shown in FIG. 9. The structure of cholesteryl ester with deuterium labels at the n6 position, and the fatty acid "R" acylated to the hydroxyl group is shown in FIG. 9A. The structure of cholesteryl ester with deuterium labels at the n6 position, and linoleate (18:2n6), as the exemplary fatty acid, acylated to the hydroxyl group is shown in FIG. 9B. The structure of eight fatty acids (16:0, 16:1n7, 18:1n9, 18:2n6, 20:3n6, 20:4n6, 20:5n3, 22:6n3) in the fatty acid mix for use in acylating the hydroxyl group of cholesteryl ester are shown in FIG. 9C.

[00145] Free fatty acid internal standards were produced by synthesizing a 50:50 mixture consisting of 50% deuterium-labeled fatty acid and 50% single odd-chain fatty acid. The deuterium-labeled fatty acid may be, for example, palmitate (16:0), and the odd-chain fatty acid may be, for example, 17:1n7.

[00146] For example, for free fatty acids, the composition of fatty acids are listed in Table 8. The structures of the free fatty acids comprising the mixture are shown in FIG. 10. The structure of the free fatty acid palmitate (16:0) with deuterium labels is shown in FIG. 10A. The structure of the free fatty acid, 17:1n7, is shown in FIG. 10B.

Example 3. Validating and Assessing Performance of Lipid Internal Standard Mixtures

[00147]   Lipids were extracted from samples using a methanol: dichloromethane extraction procedure. Internal standards in dichloromethane:methanol (50:50) were added to the extract solution, followed by a series of centrifugation and bottom layer recovery steps. The combined bottom layer aliquots were concentrated under nitrogen and reconstituted in 0.25mL of 10mM ammonium acetate dichloromethane:methanol (50:50). The extracts were transferred to inserts and placed in vials for analysis using infusion-MS. Infusion-MS analysis is performed on a Shimazdu LC with nano PEEk tubing combined with the Sciex SelexIon and 5500 QTRAP. The samples were analyzed via both positive and negative mode electrospray. The 5500 QTRAP scan is performed in MRM mode with the total of +1100 MRMs including internal standards.

[00148]   An exemplary internal standard mixture containing the components indicated in Table 9 was synthesized, and the synthetic internal standards mixture was evaluated to determine the concentrations and purity of the mixture. Labeled and unlabeled standard mixtures were reported to be 10.3 mgs/mL. Actual concentrations of the internal standards in the mixture were determined based on NMR and quantitative fatty acid analysis; results of these two analyses were within 2% of each other. Column 2 of Table 9 indicates the target concentration for each internal standard component of the mixture, and Columns 3 and 4 of Table 9 shows the actual concentration for each indicated component. Column 3 shows the concentration obtained for the labeled standards and Column 4 shows the concentration obtained for the unlabeled standards. A complete fatty acid analysis of the samples determined that the internal standard mixture was >99% pure.

Table 9. Composition of one exemplary internal standard mixture

Component	Target Concentration	Actual Concentration (Labeled)	Actual Concentration (Unlabeled)
PCd16:0/16:1	5%	5.7%	5.7%
PCd16:0/18:1	20%	17.9%	18.1%
PCd16:0/18:2	20%	20.9%	20.0%
PCd16:0/18:3	5%	5.7%	6.0%
PCd16:0/20:3	5%	4.6%	4.5%
PCd16:0/20:4	20%	19.7%	19.2%
PCd16:0/20:5	5%	5.2%	4.3%
PCd16:0/22:4	5%	4.1%	4.9%
PCd16:0/22:5	5%	5.3%	5.6%
PCd16:0/22:6	10%	11.0%	11.7%

[00149]   The performance of the exemplary labeled and unlabeled internal standard mixtures was assessed to determine if the presence of the label caused a shift in chromatographic retention time. The labeled standards did not substantially shift retention time compared to unlabeled analogs.

[00150]   The quantification performance of the labeled and unlabeled internal standard mixtures was assessed experimentally in a solution of unlabeled standards and in serum. Lipids were extracted from an unlabeled internal standard mixture sample and a serum sample, each in the presence of labeled internal standard mixture and PC17:0/17:0 (a traditional lipidomics internal standard). The samples were extracted five times to produce a total of ten samples (5 serum and 5 unlabeled standard mixture). The lipids extracted from the samples were analyzed using described methods. The concentration of each phosphatidylcholine species was calculated in nMoles per mL of starting material. Concentrations were calculated using both the internal standard mixture and the traditional internal standard PC17:0/17:0. In serum, the precision as determined by calculating the analytical CV was 20% and 11% for the traditional PC17:0/17:0 IS and the IS mixture respectively. In samples of the unlabeled internal standard mixture, the overall precision as determined by calculating the analytical CV was 11% and 3% for the traditional PC17:0/17:0 IS and the IS mixture respectively.

[00151]   The Mole% composition for the fatty acids was calculated using the IS mixture and the traditional IS and compared to the actual concentration of the fatty acids in the sample. The

calculated values obtained with the IS mixture were closer to the actual values than those values calculated using the traditional IS. The results are graphically presented in FIG. 11.

Example 4. Assessing Internal Standard Mixture in Human Samples

[00152]   To assess the quantification performance of the internal standard mixture, the lipid molecular species in 15 human serum samples, run in triplicate, were measured using the mixture of internal standards described in Table 1. Lipids were extracted from the serum samples in the presence of the labeled internal standard mixture and the lipid extracts were analyzed using the described methods. The mean concentration of each of the 1,100 measured lipid molecular species in the triplicate samples was calculated using the internal standard mixture, and the precision was determined by calculating the analytical CV. The average concentration and CV values for the 15 triplicate samples were plotted for all 1,100 measured lipid molecular species. The scatterplot showing the relationship of the %CV to the concentration of lipid molecular species is graphically presented in FIG. 12. The CV was lower for lipid molecular species at the highest concentrations, however CV as low as 10% was obtained for certain molecular species at very low concentrations (e.g., 0.001 uM)..

[00153]   The average concentration of the CE lipid class from all serum samples was 3676.72, and the average CV was 1.22%. These results are presented in box plot format in FIG. 13. The signal, which is the ratio of the concentration of CE lipid class in the serum samples to the blanks, was 1729.02. The average concentration of the TAG lipid class was 4105.8, and the average CV was 2.79%. These results are presented in box plot format in FIG. 14. The signal for the TAG lipid class was 1348.33.

[00154]   In another example, the performance of the internal standard mixture compared to a traditional internal standard was evaluated in 25 human plasma samples. Using the methods described herein and the composition of internal standards described in Table 1, the fatty acid composition of three exemplary lipid classes (CE, PC, and PE) was calculated. These results were compared to calculations done using the traditional method of a single internal standard per lipid class and were also compared to quantitative values.

[00155]   For quantitation using a single internal standard for each lipid class (traditional method), the dCE(16:0) internal standard was used for cholesteryl ester, the dPC(16:0/18:1) internal standard was used for phosphatidylcholine, and the dPE(18:0/18:1) internal standard was used for phosphatidylethanolamine. For single internal standard quantitation, fatty acids within

CE, PC or PE lipid classes were assigned to the single CE, PC or PE internal standard, respectively.

[00156]   For quantitation using an exemplary mixture of internal standards, the full complement of internal standards for each lipid class was used (Table 1). In all cases, the lipid molecular species to be quantified was assigned to an internal standard with the same number of double bonds in the fatty acid composition. For instance, the molecular species PC(16:0/22:5), PC(18:0/22:5) and PC(14:0/22:5) were all assigned to the internal standard dPC(16:0/22:5).

[00157]   Total concentrations of cholesteryl ester (CELC), phosphatidylcholine (PCLC) and phosphatidylethanolamine (PELC) lipid classes were determined from the 25 human plasma samples with calculations using both a single internal standard and a mixture of internal standards (Multiple IS) per lipid class (FIG. 15). Results were compared to values obtained by quantitative GC-FID analysis (TrueMass, described elsewhere). Bias was calculated as:

$$((\text{Calculated value - TrueMass value})/\text{TrueMass value}) * 100$$

[00158]   Bias was calculated for the single internal standard method and the multiple internal standard method. The results are presented in Table 10.

Table 10. Bias for total concentration calculated for three exemplary lipid classes

	Single IS	Multiple IS
Cholesteryl Ester (CELC)	280.2%	15%
Phosphatidylcholine (PCLC)	-18.1%	-4.5%
Phosphatidylethanolamine (PELC)	-29.8%	-6.1%

[00159]   Next, the fatty acid composition of CE, PC, and PE lipid classes was determined. All values were expressed in Mole%, a form of data that provides the relative amount of a fatty acid present in each class relative to all fatty acids. As one example, a comparison of the fatty acid composition of cholesteryl ester (CE) for the single IS (dCE(16:0)) and multiple IS methods relative to quantitative (TrueMass) values is shown in FIG. 16. The y=x line indicates a value with a bias equal to zero. All 25 subjects are plotted for each of the 22 fatty acids measured in the CE lipid class. Although some fatty acids are measured effectively using a single internal

standard, others show significant bias, however, the bias is lower when the multiple IS were used.

[00160]   In particular, the polyunsaturated fatty acids appeared to be substantially biased using the single IS method. Specific examples of the bias against polyunsaturated fatty acids from the CE and PC lipid classes are shown below. The bias was calculated as described above and is presented in Table 11. The true (quantitative) value (μM) and the estimated value (calculated using an internal standard) (μM) for the exemplary fatty acids of the PC and CE lipid classes were plotted for each of the 25 plasma samples. The results are shown in FIG. 17.

Table 11. Bias for relative amount of exemplary fatty acids in the given lipid class

	Single IS	Multiple IS
PC20:5	-49.8%	-5.3%
PC22:6	-80.9%	-7.3%
CE20:4	67.7%	-2.9%
CE20:5	124.1%	6.8%

[00161]   Here we show that using a single internal standard per class can cause quantitative and compositional biases in the results, because the single internal standard was not able to accurately quantify all molecular species within a class. Using the methods described herein, the quantitative and compositional bias in lipidomics assays was significantly and substantially reduced.

[00162]   Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[00163]   One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

THAT WHICH IS CLAIMED:

1. A method for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising:

    attaching an isotopically-labeled fatty acid at a first position on a lipid backbone through an acylation reaction for a lipid class having at least two acyl groups; and

    attaching a mixture of at least two different fatty acids to the lipid backbone at a separate position through an acylation reaction,

    wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

2. The method of claim 1, wherein the lipid class comprises triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters.

3. The method of claim 1, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

4. The method of claim 1, wherein the first position on the lipid backbone is a sn-1 position, a sn-2 position or a sn-3 position.

5. The method of claim 1, wherein the first position on the lipid backbone is a sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

6. The method of claim 1, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

7. The method of claim 1, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

8. The method of claim 1, wherein the lipid class consists of phosphatidylcholines and o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate

position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein a deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

9. The method of claim 8, wherein the mixture of fatty acidsat the separate position are present at the ratios shown in Table 2.

10. The method of claim 8, wherein the sample comprises a human sample.

11. The method of claim 1, wherein the lipid class consists of phosphatidylethanolamines and p-phosphatidylethanolamines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein a deuterium-labeled octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of fatty acids at the separate position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

12. The method of claim 11, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 3.

13. The method of claim 11, wherein the sample comprises a human sample.

14. The method of claim 1, wherein the lipid class consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

15. The method of claim 14, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 4.

16. The method of claim 14, wherein the sample comprises a human sample.

17. The method of claim 1, wherein the lipid class consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

18. The method of claim 17, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 6.

19. The method of claim 17, wherein the sample comprises a human sample.

20. The method of claim 1, wherein the lipid class consists of triacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 or sn-3 position, or wherein the first position is a sn-3 position and the separate position is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, wherein oleate (18:1n9) is present at a separate position, and wherein the mixture of fatty acid groups at another separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

21. The method of claim 20, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 5.

22. The method of claim 20, wherein the sample comprises a human sample.

23. The method of claim 1, wherein the sample of interest is a biological sample.

24. The method of claim 23 wherein the biological sample comprises a plant sample, animal sample, or human sample.

25. The method of claim 23, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

26. The method of claim 1, further comprising performing quantitative fatty acid analysis to quantify the amount of each of the fatty acids in the mixture.

27. The method of claim 1, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

28. A method for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising:

    attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group,

wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

29. The method of claim 28, wherein the lipid class comprises triacylglycerols, diacylglycerols, free fatty acids, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

30. The method of claim 28, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

31. The method of claim 28, wherein the lipid class consists of cholesteryl esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group comprises hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-

octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3).

32. The method of claim 31, wherein the mixture of fatty acids at the hydroxyl group are present at the ratios shown in Table 7.

33. The method of claim 31, wherein the sample of interest is a biological sample.

34. The method of claim 33, wherein the biological sample comprises a human sample.

35. The method of claim 28, wherein the sample of interest is a biological sample.

36. The method of claim 28, wherein the biological sample comprises a plant, animal or human sample.

37. The method of claim 36, wherein the sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

38. The method of claim 28, further comprising performing quantitative fatty acid analysis to quantify the amount of each of the fatty acids in the mixture.

39. The method of claim 28, wherein the fatty acid is a saturated fatty acid.

40. A composition for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising:

    a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups; and

    a mixture of at least two different fatty acids present at a separate position on the lipid backbone,

wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

41. The composition of claim 40, wherein the lipid class comprises triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters.

42. The composition of claim 40, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

43. The composition of claim 40, wherein the first position on the lipid backbone is a sn-1 position, a sn-2 position or a sn-3 position.

44. The composition of claim 40, wherein the first position on the lipid backbone is a sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

45. The composition of claim 40, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

46. The composition of claim 40, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

47. The composition of claim 40, wherein one of the lipid classes consists of phosphatidylcholines or o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

48. The composition of claim 47, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 2.

49. The composition of claim 47, wherein the sample comprises a human sample.

50. The composition of claim 40, wherein one of the lipid classes consists of phosphatidylethanolamines or p-phosphatidylethanolamines, wherein the first position is

a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of fatty acids at the separate position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

51. The composition of claim 50, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 3.

52. The composition of claim 50, wherein the sample of interest is a biological sample.

53. The composition of claim 52, wherein the biological sample comprises a human sample.

54. The composition of claim 40, wherein one of the lipid classes consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

55. The composition of claim 54, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 4.

56. The composition of claim 54, wherein the sample of interest is a biological sample.

57. The composition of claim 56, wherein the biological sample comprises a human sample.

58. The composition of claim 40, wherein one of the lipid classes consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

59. The composition of claim 58, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 6.

60. The composition of claim 58 wherein the sample of interest is a biological sample.

61. The composition of claim 60, wherein the biological sample comprises a human sample.

62. The composition of claim 40, wherein one of the lipid classes consists of triacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 or sn-3 position, or wherein the first position is a sn-3 position and the separate position is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, wherein oleate (18:1n9) is present at a separate position, and wherein the mixture of fatty acids at another separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

63. The composition of claim 62, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 5.

64. The composition of claim 62, wherein the sample of interest is a biological sample.

65. The composition of claim 64, wherein the biological sample comprises a human sample.

66. The composition of claim 40, wherein the sample of interest is a biological sample.

67. The composition of claim 66, wherein the biological sample of interest is a human sample.

68. The composition of claim 66, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

69. The composition of claim 40, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

70. A composition for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising:

a lipid backbone having one or more isotopic labels, wherein the lipid backbone is for a lipid class having at least one acyl group; and

a mixture of at least two different fatty acids present at a single position on the lipid backbone,

wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest.

71. The composition of claim 70, wherein the lipid class comprises triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

72. The composition of claim 70, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

73. The composition of claim 70, wherein one of the lipid classes consists of cholesteryl esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group comprises hexadecanoyl (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

74. The composition of claim 73, wherein the mixture of fatty acids at the hydroxyl group are present at the ratios shown in Table 7.

75. The composition of claim 73, wherein the sample of interest is a biological sample.

76. The composition of claim 75, wherein the biological sample comprises a human sample.

77. The composition of claim 70, wherein the sample of interest is a biological sample.

78. The composition of claim 77, wherein the biological sample comprises a human sample.

79. The composition of claim 70, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

80. A kit comprising:

    i.   one or more mixtures of lipid molecules for use as an internal standard, wherein each of the one or more mixtures of lipid molecules is representative of the composition of lipid molecular species present in each of one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising:

            a lipid backbone having an isotopically-labeled fatty acid at a first position on the lipid backbone and a mixture of at least two different fatty acids present at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups, or

            a lipid backbone having one or more isotopic labels and a mixture of at least two different fatty acids present at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group,

    wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest; and

    ii. instructions for using the one or more mixtures of lipid molecules as the internal standard for one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest.

81. The kit of claim 80, comprising two or more of the mixtures of the lipid molecules representative of the composition of lipid molecular species present in two or more of the corresponding lipid classes in the sample of interest.

82. The kit of claim 81, wherein each of the two or more mixtures of the lipid molecules are packaged as a single component.

83. The kit of claim 81, wherein each of the two or more mixtures of the lipid molecules are packaged as separate components.

84. The kit of claim 80, wherein the mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise the mixtures of fatty acids listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

85. The kit of claim 80, further comprising reagents for the detecting and quantifying.

86. The kit of claim 80, further comprising a control sample having a known concentration of the composition of lipid molecular species.

87. The kit of claim 80, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

88. The kit of claim 80, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

89. The kit of claim 80, wherein the sample of interest is a biological sample.

90. The kit of claim 89, wherein the biological sample comprises a human sample.

91. The kit of claim 89, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

92. The kit of claim 80, further comprising a mixture of free fatty acids for use as an internal standard that is representative of the composition of free fatty acids in the sample of interest.

93. The kit of claim 80, wherein the lipid class having at least two acyl groups comprises triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, or wax diesters.

94. The kit of claim 80, wherein the first position on the lipid backbone is the sn-1 position, sn-2 position or sn-3 position.

95. The kit of claim 80, wherein the first position on the lipid backbone is a sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

96. The kit of claim 80, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

97. The kit of claim 80, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

98. The kit of claim 80, wherein one of the lipid classes consists of phosphatidylcholines or o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

99. The kit of claim 98, wherein the mixture of fatty acids at the first position are present at the ratios shown in Table 2.

100.   The kit of claim 98, wherein the sample of interest is a biological sample.

101.   The kit of claim 100, wherein the biological sample comprises a human sample.

102.   The kit of claim 80, wherein one of the lipid classes consists of phosphatidylethanolamines or p-phosphatidylethanolamines, wherein the first position is a sn-1 position and the separate position is a sn-2 position or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of fatty acids at the separate position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

103.   The kit of claim 102, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 3.

104.   The kit claim of 102, wherein the sample of interest is a biological sample.

105.   The kit claim of 104, wherein the biological sample comprises a human sample.

106.   The kit of claim 80, wherein one of the lipid classes consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the

mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

107.   The kit of claim 106, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 4.

108.   The kit of claim 106, wherein the sample of interest is a biological sample.

109.   The kit of claim 109, wherein the biological sample comprises a human sample.

110.   The kit of claim 80, wherein one of the lipid classes consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

111.   The kit of claim 110, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 6.

112.   The kit of claim 110, wherein the sample of interest is a biological sample.

113.   The kit of claim 112, wherein the biological sample comprises a human sample.

114.   The kit of claim 80, wherein one of the lipid classes consists of triacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 or sn-3 position, or wherein the first position is a sn-3 position and the separate position is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first1 position, wherein oleate (18:1n9) is present at a separate position, and wherein the mixture of fatty acids at another separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

115.   The kit of claim 114, wherein the mixture of fatty acids at the separate position are present at the ratios shown in Table 5.

116.   The kit of claim 114, wherein the sample of interest is a biological sample.

117.   The kit of claim 116, wherein the biological sample comprises a human sample.

118.   The kit of claim 80, wherein the lipid class having at least one acyl group comprises triacylglycerols, diacylglycerols, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, CDP-diacylglycerol, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax diesters cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, wax esters, 1-monoacylglycerol.

119.   The kit of claim 80, wherein one of the lipid classes consists of cholesteryl esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group comprises hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3).

120.   The kit of claim 119, wherein the mixture of fatty acids at the hydroxyl group are present at the ratios shown in Table 7.

121.   The kit of claim 119, wherein the sample of interest is a biological sample.

122.   The kit of claim 121, wherein the biological sample comprises a human sample.

123.   A method for one of detecting and quantifying lipid molecules present in a sample of interest, comprising:

          adding to a sample of interest a known amount of a composition according to claim 40 having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest; and

one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest by using the representative mixture of lipid molecules as an internal standard.

124.   The method of claim 123, wherein the sample of interest is a biological sample.

125.   The method of claim 124, wherein the biological sample comprises a human sample.

126.   The method of claim 123, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS), high performance liquid chromatography (HPLC), isocratic HPLC, gradient HPLC, normal phase chromatography, reverse phase HPLC, size exclusion chromatography, ion exchange chromatography, capillary electrophoresis, microfluidics, chromatography, gas chromatography (GC), or thin-layer chromatography (TLC), and combinations thereof.

127.   The method of claim 123, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS).

128.   A method for one of detecting and quantifying lipid molecules present in a sample of interest, comprising:

adding to a sample of interest a known amount of a composition according to claim 70 having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest; and

one of detecting and quantifying the lipid molecular species present in the corresponding lipid class in the sample of interest by using the representative mixture of lipid molecules as an internal standard.

129.   The method of claim 128, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS), high performance liquid chromatography (HPLC), isocratic HPLC, gradient HPLC, normal phase chromatography, reverse phase HPLC, size exclusion chromatography, ion exchange chromatography, capillary electrophoresis, microfluidics,

chromatography, gas chromatography (GC), or thin-layer chromatography (TLC), and combinations thereof.

130.   The method of claim 128, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS).

131.   The method of claim 128, wherein the sample of interest is a biological sample.

132.   The method of claim 131, wherein the biological sample comprises a human sample.

133.   A method for synthesizing one or more mixtures of lipid molecules for use as an internal standard representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising one or more of:

    attaching a mixture of at least two different isotopically-labeled fatty acids to a lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group;

    attaching a mixture of at least two different fatty acids to an isotopically-labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; or

    attaching a single isotopically-labeled fatty acid to a lipid backbone at a first position through an acylation reaction for a lipid class having at least two fatty acids and attaching a mixture of at least two different fatty acids to a separate position on the lipid backbone through an acylation reaction,

wherein the mixture of the at least two different fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest.

134.   The method of claim 133, wherein the lipid class comprises one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidylethanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine,

lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

135.   The method of claim 133, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

136.   The method of claim 133, wherein the first position on the lipid backbone is a sn-1 position, a sn-2 position, or a sn-3 position.

137.   The method of claim 133, wherein the first position on the lipid backbone is an sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

138.   The method of claim 133, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

139.   The method of claim 133, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

140.   The method of claim 133, wherein one of the lipid classes consists of phosphatidylcholines or o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein a deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acid groups at the separate position or at the single position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

141.   The method of claim 140, wherein the mixture of fatty acids is present at the ratios shown in Table 2.

142.   The method of claim 140, wherein the sample comprises a human sample.

143.   The method of claim 133, wherein one of the lipid classes consists of phosphatidylethanolamines or p-phosphatidylethanolamines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is

a sn-2 position and the separate position is a sn-1 position, wherein a deuterium-labeled octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of fatty acid groups at the separate position or at the single position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

144.   The method of claim 143, wherein the mixture of fatty acids are present at the ratios shown in Table 3.

145.   The method of claim 143, wherein the sample comprises a human sample.

146.   The method of claim 133, wherein one of the lipid classes consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

147.   The method of claim 146, wherein the mixture of fatty acids are present at the ratios shown in Table 4.

148.   The method of claim 146, wherein the sample comprises a human sample.

149.   The method of claim 133, wherein one of the lipid classes consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acid groups at the separate position or at the single position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

150.   The method of claim 149, wherein the mixture of fatty acids is present at the ratios shown in Table 6.

151.   The method of claim 149, wherein the sample comprises a human sample.

152.   The method of claim 133, wherein one of the lipid classes consists of triacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 or sn-3 position, or wherein the first position is a sn-3 position and the separate position is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position or at the single position, wherein oleate (18:1n9) is present at a separate position, and wherein the mixture of fatty acid groups at another separate position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

153.   The method of claim 152, wherein the mixture of fattys is present at the ratios shown in Table 5.

154.   The method of claim 153, wherein the sample comprises a human sample.

155.   The method of claim 133, wherein one of the lipid classes consists of cholesteryl esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group comprises hexadecanoate (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoate (22:6n3).

156.   The method of claim 155, wherein the mixture of fatty acids at the hydroxyl group is present at the ratios shown in Table 7.

157.   The method of claim 155, wherein the sample comprises a human sample.

158.   The method of claim 133, wherein the sample of interest comprises a biological sample.

159.   The method of claim 158, wherein the biological sample comprises a plant sample, animal sample, or human sample.

160.   The method of claim 158, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

161.	The method of claim 133, further comprising performing quantitative fatty acid analysis to quantify the amount of each of the mixture of fatty acids.

162.	The method of claim 133, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

163.	A composition for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of:

a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group;

an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or

a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids,

wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acids in the lipid molecular species present in the corresponding lipid class in the sample of interest.

164.	The composition of claim 163, wherein the lipid class comprises one or more of triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids, phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol,

phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide, phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

165.   The composition of claim 163, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

166.   The composition of claim 163, wherein the first position on the lipid backbone is the sn-1 position, the sn-2 position or the sn-3 position.

167.   The composition of claim 163, wherein the first position on the lipid backbone is a sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

168.   The composition of claim 163, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

169.   The composition of claim 163, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

170.   The composition of claim 163, wherein one of the lipid classes consists of phosphatidylcholines or o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acid groups at the separate position or at the single position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

171.   The composition of claim 170, wherein the mixture of fatty acids is present at the ratios shown in Table 2.

172.   The composition of claim 170, wherein the sample comprises a human sample.

173.   The composition of claim 163, wherein one of the lipid classes consists of phosphatidylethanolamines or p-phosphatidylethanolamines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of

fatty acids at the separate position or at the single position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

174.   The composition of claim 173, wherein the mixture of fatty acids is present at the ratios shown in Table 3.

175.   The composition of claim 174, wherein the sample of interest is a biological sample.

176.   The composition of claim 175, wherein the biological sample comprises a human sample.

177.   The composition of claim 163, wherein one of the lipid classes consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

178.   The composition of claim 177, wherein the mixture of fatty acids is present at the ratios shown in Table 4.

179.   The composition of claim 177 wherein the sample of interest is a biological sample.

180.   The composition of claim 179, wherein the biological sample comprises a human sample.

181.   The composition of claim 163, wherein one of the lipid classes consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

182.   The composition of claim 181, wherein the mixture of fatty acids is present at the ratios shown in Table 6.

183.   The composition of claim 181, wherein the sample of interest is a biological sample.

184.   The composition of claim 183, wherein the biological sample comprises a human sample.

185.   The composition of claim 163, wherein one of the lipid classes consists of triacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 or sn-3 position, or wherein the first position is a sn-3 position and the separate position is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, wherein oleate (18:1n9) is present at a separate position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

186.   The composition of claim 185, wherein the mixture of fatty acids is present at the ratios shown in Table 5.

187.   The composition of claim 185, wherein the sample of interest is a biological sample.

188.   The composition of claim 187, wherein the biological sample comprises a human sample.

189.   The composition of claim 163, wherein the sample of interest is a biological sample.

190.   The composition of claim 189, wherein the biological sample of interest is a human sample.

191.   The composition of claim 189, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

192.   The composition of claim 163, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

193.   The composition of claim 163, wherein one of the lipid classes consists of cholesteryl esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty acids attached to the hydroxyl group comprises hexadecanoyl (16:0), 9Z-hexadecenoate (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

194.   The composition of claim 193, wherein the mixture of fatty acids is present at the ratios shown in Table 7.

195.   The composition of claim 193, wherein the sample of interest is a biological sample.

196.   The composition of claim 195, wherein the biological sample comprises a human sample.

197.   A kit comprising:

i.   one or more mixtures of lipid molecules for use as an internal standard, wherein each of the one or more mixtures of lipid molecules is representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, each mixture of lipid molecules comprising one or more of:

a lipid backbone having a mixture of at least two different isotopically-labeled fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group;

an isotopically-labeled lipid backbone having a mixture of at least two different fatty acids at a single position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least one acyl group; or

a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two acyl groups,

wherein the mixture of at least two different fatty acids is representative of the

fatty acids that occur in the corresponding lipid class in the sample of interest,

and wherein each of the fatty acids in the mixture is present at a ratio

representative of the ratio of occurrence of the fatty acids in the lipid

molecular species present in the corresponding lipid class in the sample of

interest; and

ii. instructions for using the one or more mixtures of lipid molecules as the internal

standard for one of detecting and quantifying the lipid molecular species present in the

lipid class in the sample of interest.

198.   The kit of claim 197, comprising two or more of the mixtures of the lipid

molecules representative of the composition of lipid molecular species present in two or

more corresponding lipid classes in the sample of interest.

199.   The kit of claim 198, wherein each of the two or more mixtures of the lipid

molecules are packaged as a single component.

200.   The kit of claim 198, wherein each of the two or more mixtures of the lipid

molecules are packaged as separate components.

201.   The kit of claim 197, wherein the lipid class comprises one or more of

triacylglycerols, diacylglycerols, 1-monoacylglycerol, phospholipids,

phosphatidylcholine, o-phosphatidylcholine, phosphatidyletanolamine, p-

phosphatidylethanolamine, cholesteryl esters, lysophosphatidylcholine,

lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol,

lysophosphatidylglycerol, lysophosphatidic acid, lyso CDP-diacylglycerol, CDP-

diacylglycerol, sphingomyelin, cardiolipin, phosphatidylserine, phosphatidylinositol,

phosphatidylglycerol, phosphatidic acid, ceramide, lactosylceramide, glucosylceramide,

phytoceramide, 6-hydroxyceramide, cerebroside, ganglioside, wax esters, or wax diesters.

202.   The kit of claim 197, wherein one or more of the mixtures of fatty acids in each of

the one or more mixtures of lipid molecules comprise the mixtures of lipid molecular

species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or

Table 7.

203.   The kit of claim 197, further comprising reagents for the detecting and

quantifying.

204.   The kit of claim 197, further comprising a control sample having a known concentration of the composition of lipid molecular species.

205.   The kit of claim 197, wherein the isotopic label comprises ^{2}H, ^{13}C, or ^{15}N.

206.   The kit of claim 197, wherein the first position on the lipid backbone is the sn-1 position, sn-2 position or sn-3 position.

207.   The kit of claim 197, wherein the first position on the lipid backbone is a sn-1 position and the separate position on the lipid backbone is a sn-2 or a sn-3 position.

208.   The kit of claim 197, wherein the first position on the lipid backbone is a sn-2 position and the separate position on the lipid backbone is a sn-1 or a sn-3 position.

209.   The kit of claim 197, wherein the first position on the lipid backbone is a sn-3 position and the separate position on the lipid backbone is a sn-1 or a sn-2 position.

210.   The kit of claim 197, wherein one of the lipid classes consists of phosphatidylcholines or o-phosphatidylcholines, wherein the first position is a sn-1 position and the separate position is a sn-2 position, or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled hexadecanoyl-d9 (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises 9Z-hexadecenoyl (16:1n7), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z-docosatetraenoyl (22:4n6), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

211.   The kit of claim 210, wherein the mixture of fatty acids is present at the ratios shown in Table 2.

212.   The kit of claim 210, wherein the sample of interest comprises a biological sample.

213.   The kit of claim 212, wherein the biological sample comprises a human sample.

214.   The kit of claim 197, wherein one of the lipid classes consists of phosphatidylethanolamines or p-phosphatidylethanolamines, wherein the first position is a sn-1 position and the separate position is a sn-2 position or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled

octadecanoyl-d9 (18:0-d9) is present at the first position , and wherein the mixture of fatty acids at the separate position or at the single position comprises 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), 7Z,10Z,13Z,16Z,19Z-docosapentaenoyl (22:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

215.   The kit of claim 214, wherein the mixture of fatty acids is present at the ratios shown in Table 3.

216.   The kit claim of 214, wherein the sample of interest comprises a biological sample.

217.   The kit claim of 216, wherein the biological sample comprises a human sample.

218.   The kit of claim 197, wherein one of the lipid classes consists of sphingomyelins, wherein deuterium-labeled sphingosine is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), 9Z-octadecenoyl (18:1n9), tetracosanoyl (24:0), and 15Z-tetracosenoyl (24:1n9).

219.   The kit of claim 218, wherein the mixture of fatty acids is present at the ratios shown in Table 4.

220.   The kit of claim 218, wherein the sample of interest is a biological sample.

221.   The kit of claim 220, wherein the biological sample comprises a human sample.

222.   The kit of claim 197, wherein one of the lipid classes consists of diacylglycerols, wherein the first position is a sn-1 position and the separate position is a sn-2 position or wherein the first position is a sn-2 position and the separate position is a sn-1 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the first position, and wherein the mixture of fatty acids at the separate position or at the single position comprises hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

223.   The kit of claim 222, wherein the mixture of fatty acids is present at the ratios shown in Table 6.

224.   The kit of claim 222, wherein the sample of interest comprises a biological
sample.

225.   The kit of claim 224, wherein the biological sample comprises a human sample.

226.   The kit of claim 197, wherein one of the lipid classes consists of triacylglycerols,
wherein the first position is a sn-1 position and the separate position is a sn-2 or sn-3
position, or wherein the first position is a sn-2 position and the separate position is a sn-1
or sn-3 position, or wherein the first position is a sn-3 position and the separate position
is a sn-1 or sn-2 position, wherein deuterium-labeled palmitate (16:0-d9) is present at the
first1 position, wherein oleate (18:1n9) is present at a separate position, and wherein the
mixture of fatty acids at the separate position or at the single position comprises
hexadecanoyl (16:0), octadecanoyl (18:0), 9Z-octadecenoyl (18:1n9), 9Z,12Z-
octadecadienoyl (18:2n6), 9Z,12Z,15Z-octadecatrienoyl (18:3n3), 8Z,11Z,14Z-
eicosatrienoyl (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoyl (20:4n6), and
4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl (22:6n3).

227.   The kit of claim 226, wherein the mixture of fatty acids is present at the ratios
shown in Table 5.

228.   The kit of claim 226, wherein the sample of interest comprises a biological
sample.

229.   The kit of claim 228, wherein the biological sample comprises a human sample.

230.   The kit of claim 197, wherein one of the lipid classes consists of cholesteryl
esters, wherein the single position is a hydroxyl group, and wherein the mixture of fatty
acids attached to the hydroxyl group comprises hexadecanoate (16:0), 9Z-hexadecenoate
(16:1n7), 9Z-octadecenoate (18:1n9), 9Z,12Z-octadecadienoate (18:2n6), 8Z,11Z,14Z-
eicosatrienoate (20:3n6), 5Z,8Z,11Z,14Z-eicosatetraenoate (20:4n6),
5Z,8Z,11Z,14Z,17Z-eicosapentaenoate (20:5n3), and 4Z,7Z,10Z,13Z,16Z,19Z-
docosahexaenoate (22:6n3).

231.   The kit of claim 230, wherein the mixture of fatty acids is present at the ratios
shown in Table 7.

232.   The kit of claim 230, wherein the sample of interest is a biological sample.

233.   The kit of claim 232, wherein the biological sample comprises a human sample.

234.   The kit of claim 197, wherein the isotopically-labeled fatty acid is a saturated fatty acid.

235.   The kit of claim 197, wherein the sample of interest is a biological sample.

236.   The kit of claim 235, wherein the biological sample comprises a human sample.

237.   The kit of claim 235, wherein the biological sample of interest comprises blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, a skin sample.

238.   The kit of claim 197, further comprising a mixture of free fatty acids for use as an internal standard that is representative of the composition of free fatty acids in the sample of interest.

239.   A method for one of detecting and quantifying lipid molecules present in a sample of interest, comprising:

adding to a sample of interest a known amount of a composition according to claim 163 having one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in the sample of interest; and

one of detecting and quantifying the lipid molecular species present in each of the one or more corresponding lipid classes in the sample of interest by using the composition having the one or more representative mixtures of lipid molecules as an internal standard.

240.   The method of claim 239, wherein the sample of interest is a biological sample.

241.   The method of claim 240, wherein the biological sample comprises a human sample.

242.   The method of claim 239, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS), high performance liquid chromatography (HPLC), isocratic HPLC, gradient HPLC, normal phase chromatography, reverse phase HPLC, size exclusion chromatography, ion exchange chromatography, capillary electrophoresis, microfluidics, chromatography, gas chromatography (GC), or thin-layer chromatography (TLC), and combinations thereof.

243.   The method of claim 239, wherein the method of one of detecting and quantifying the lipid molecules present in the lipid class in the sample comprises mass spectrometry (MS).

244.   The method of claim 239, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

245.   The method of claim 239, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

246.   The method of claim 40, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6.

247.   The method of claim 40, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, or Table 6.

248.   The method of claim 70, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise of one or more of the mixtures of lipid molecular species listed in Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

249.   The method of claim 70, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7

250.   The method of claim 133, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

251.   The method of claim 133, wherein the one or more mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

252.   The composition of claim 163, wherein one or more of the mixtures of fatty acids in each of the one or more mixtures of lipid molecules comprise one or more of the mixtures of lipid molecular species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

253.   The composition of claim 163, wherein one or more of the mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

254.   The kit of claim 197, wherein one or more of the mixtures of fatty acids in each of the one or more mixtures of lipid molecules consist of one or more of the mixtures of lipid molecular species listed in one or more of Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, or Table 7.

Glycerol phosphate head group

Phosphate

Glycerol

Fatty acids

Labeled palmitate (d16:0)

Mixture of unsaturated fatty acids

FIG. 1

FIG. 2

LY-PC 16:0-d9

FIG. 3

A.

PE (18:0-d9/R)

B.

PE (18:0-d9/18:1n9)

C.

18:1n9

18:2n6

18:3n3

20:3n6

20:4n6

20:5n3

22:5n3

22:6n3

FIG. 4

LY-PE 18:0-d9

FIG. 5

A.

SM (18:1n9-d9/R)

B.

SM (18:1n9-d9/16:0)

C.

16:0

18:1n9

24:0

24:1n9

FIG. 6

A.

TG (16:0-d9/18:1n9/R)

B.

TG(16:0-d9/18:1n9/16:0)

C.

16:0

18:0

18:1n9

18:2n6

18:3n3

20:3n6

20:4n6

22:6n3

FIG. 7

A.

DG (16:0-d9/R)

B.

DG (16:0-d9/16:0)

C.

16:0
18:0
18:1n9
18:2n6
18:3n3
20:4n6
20:5n3
22:6n3

FIG. 8

A.

CE-d6/R

B.

CE-d6/18:2n6

C.

16:0

18:1n7

18:1n9

18:2n6

20:3n6

20:4n6

20:5n3

22:6n3
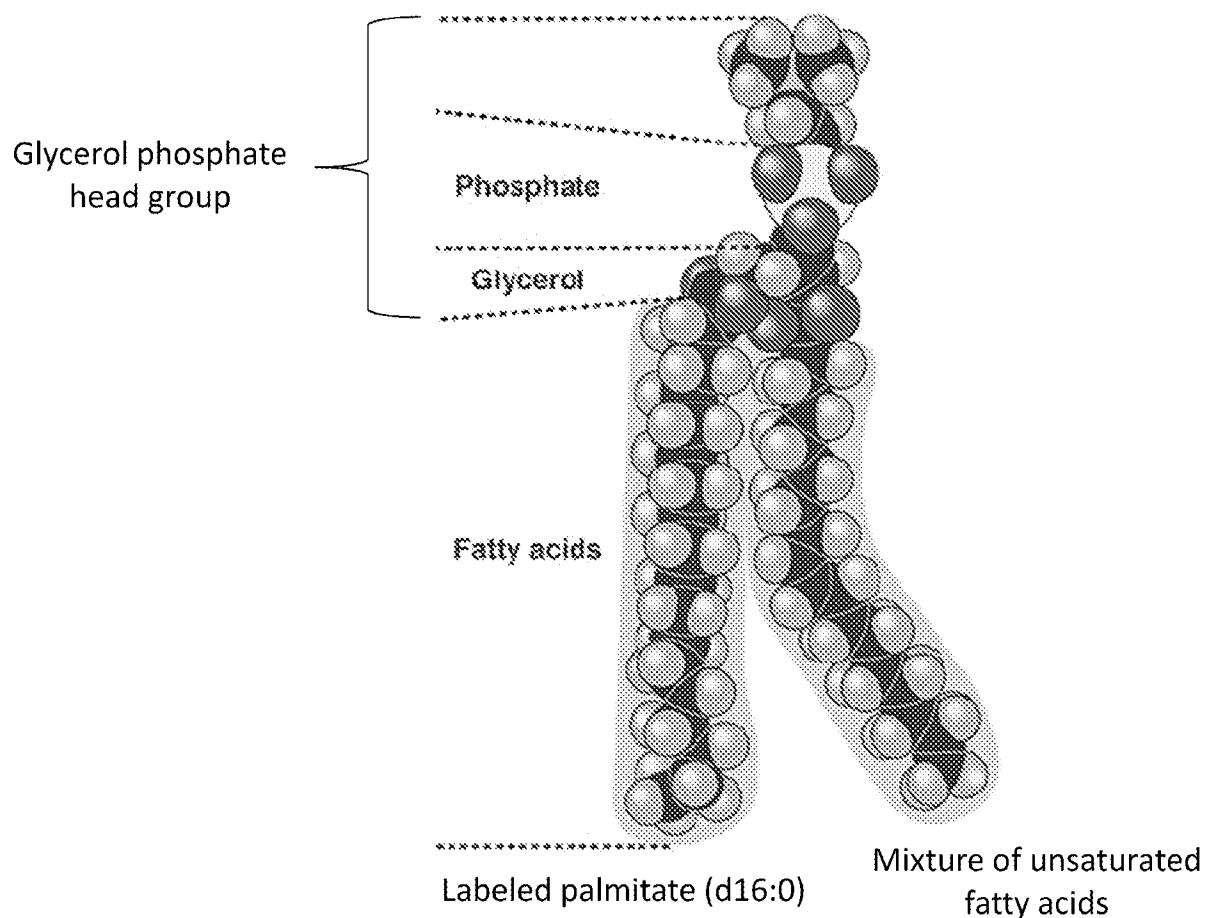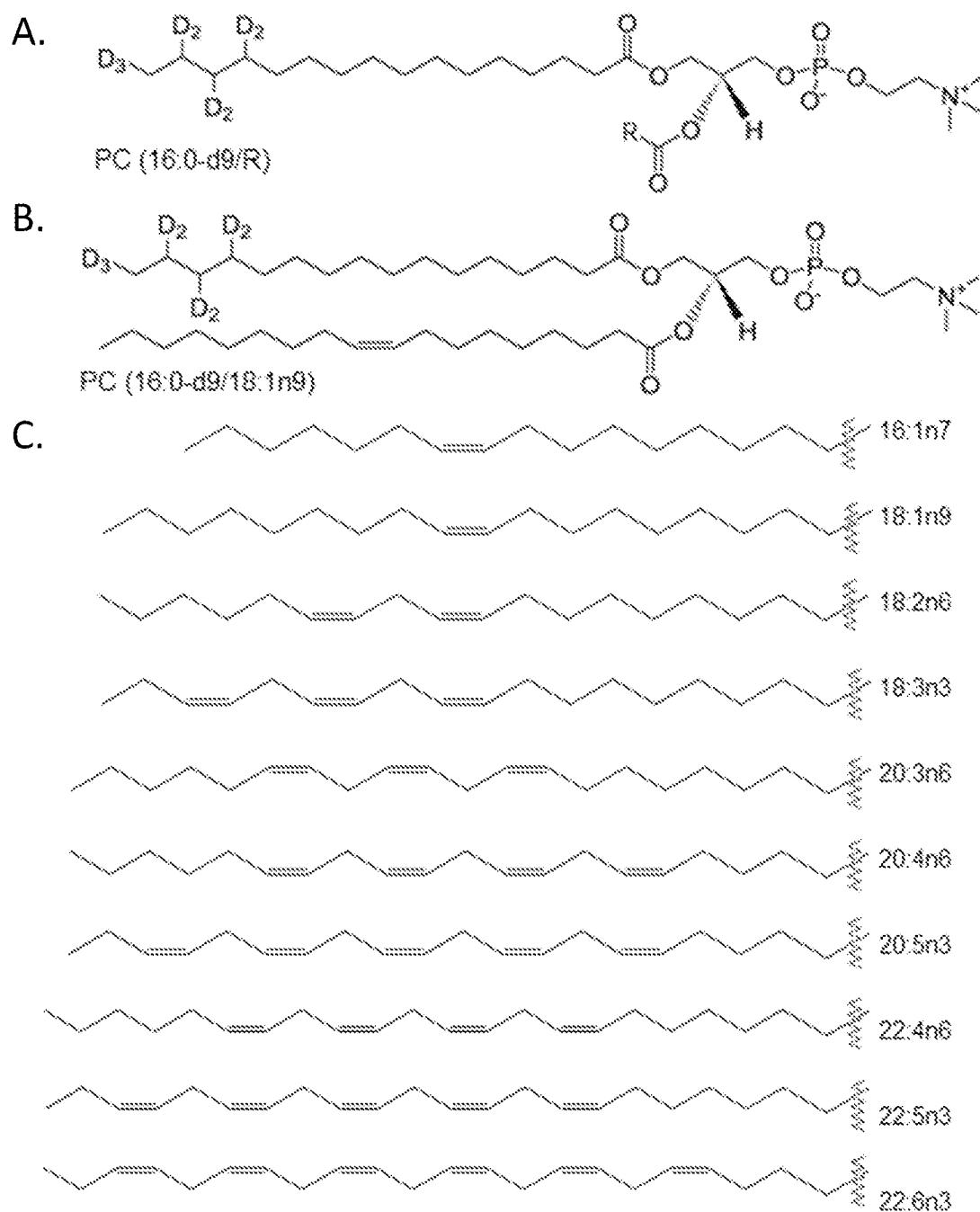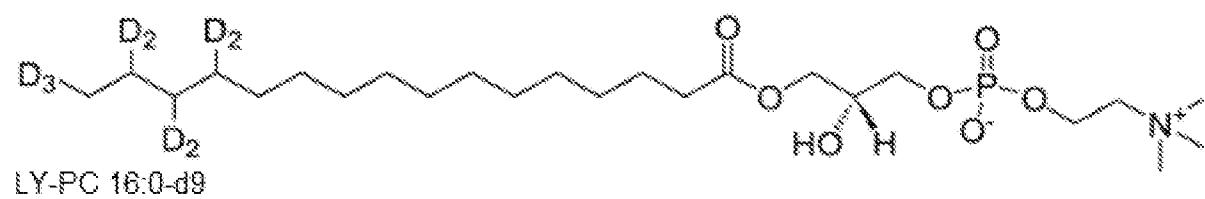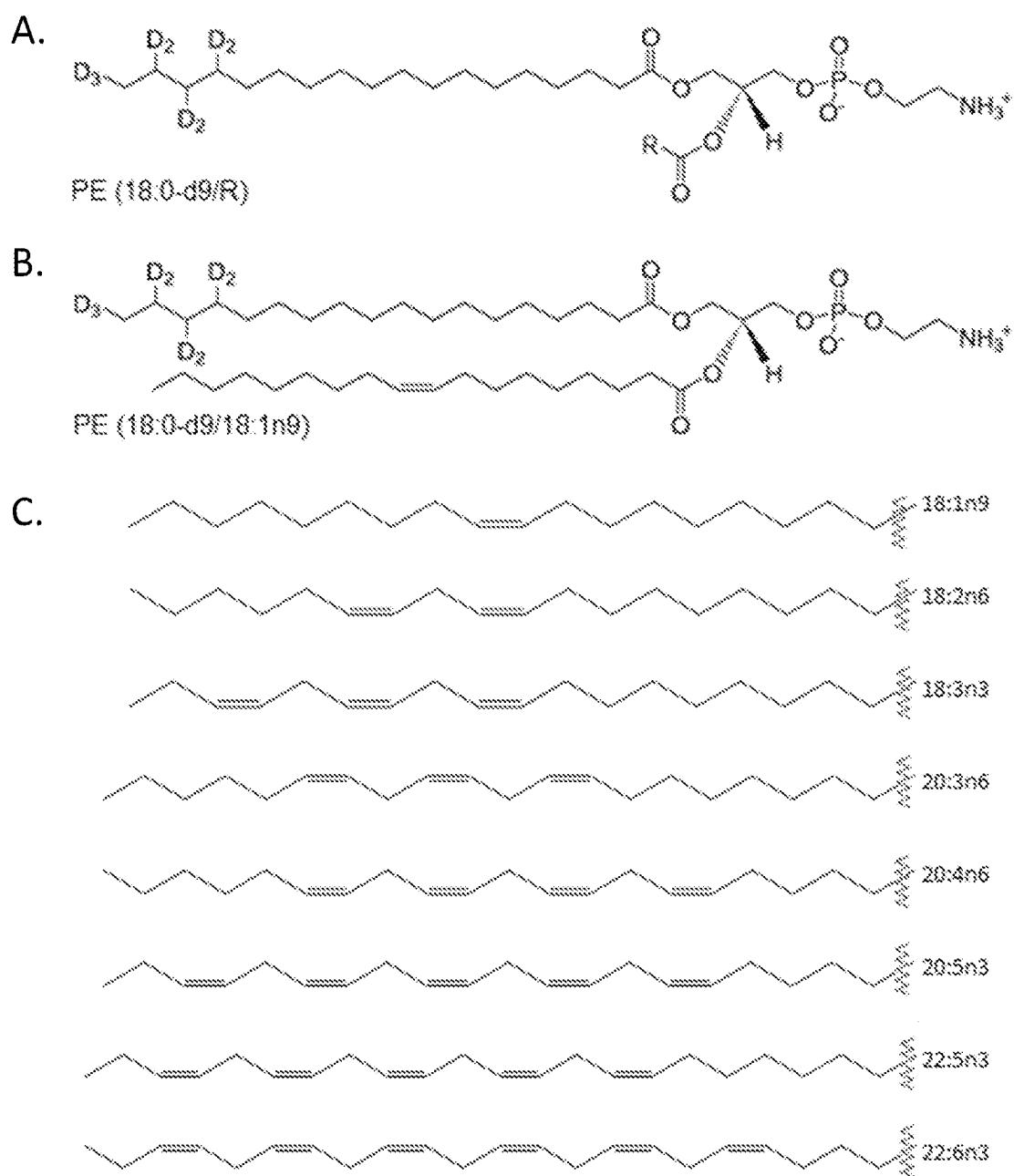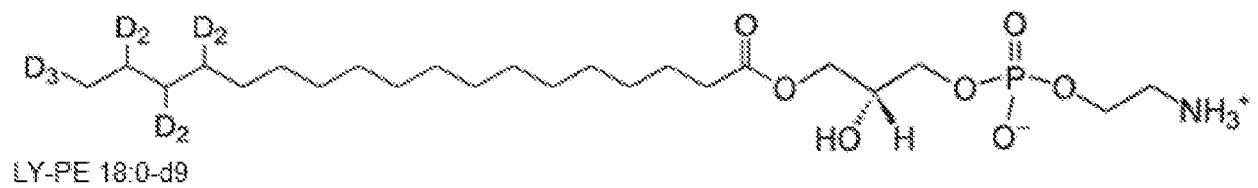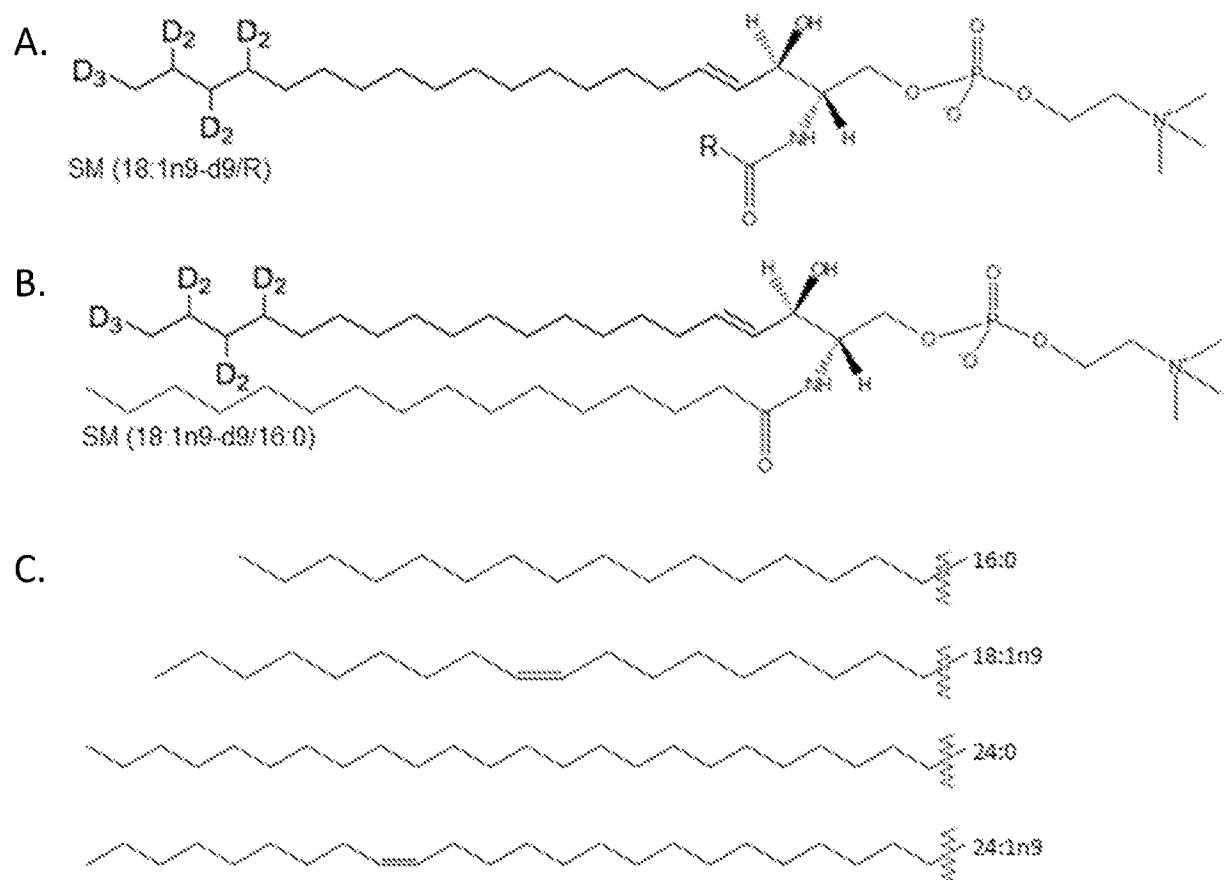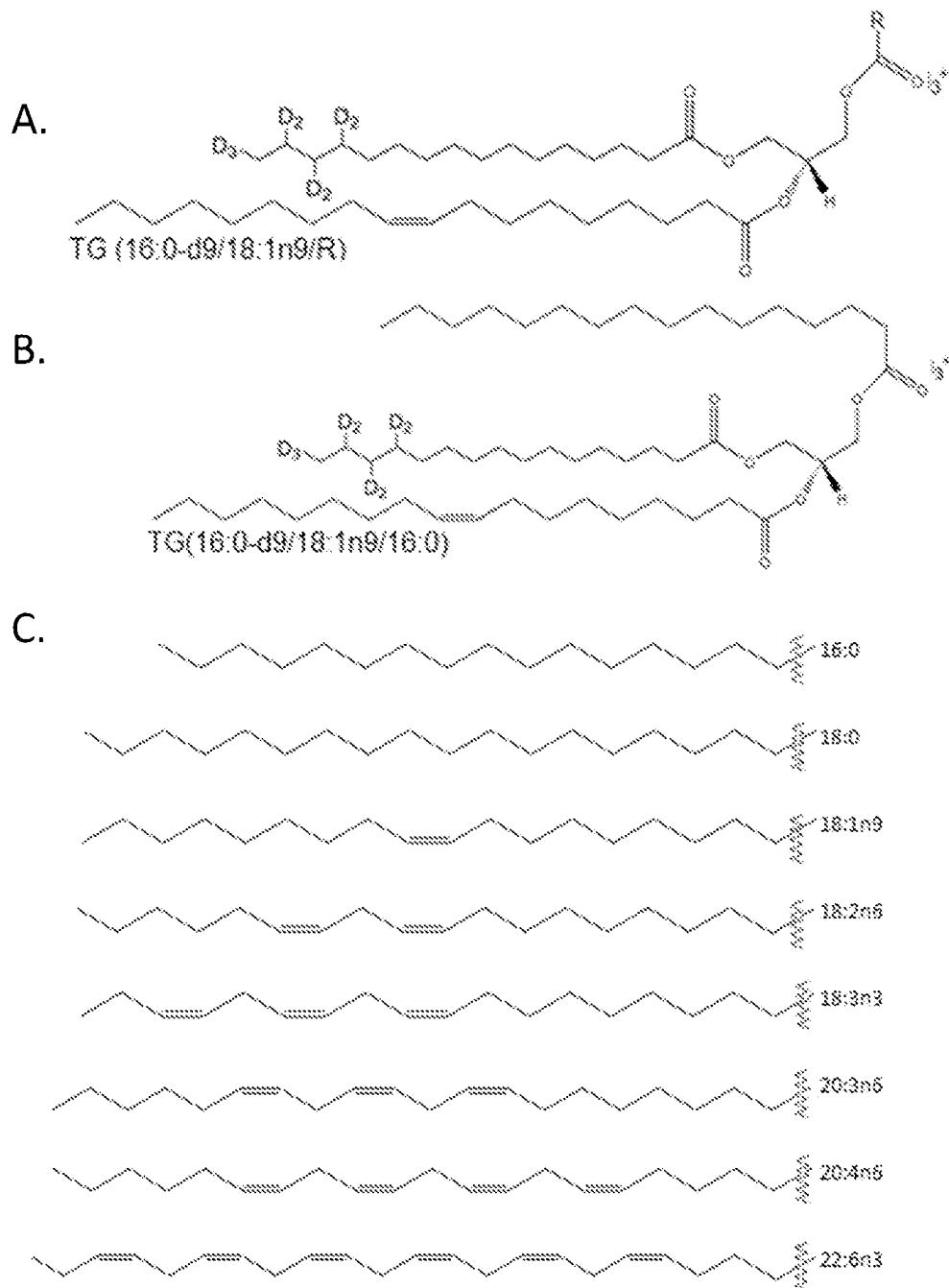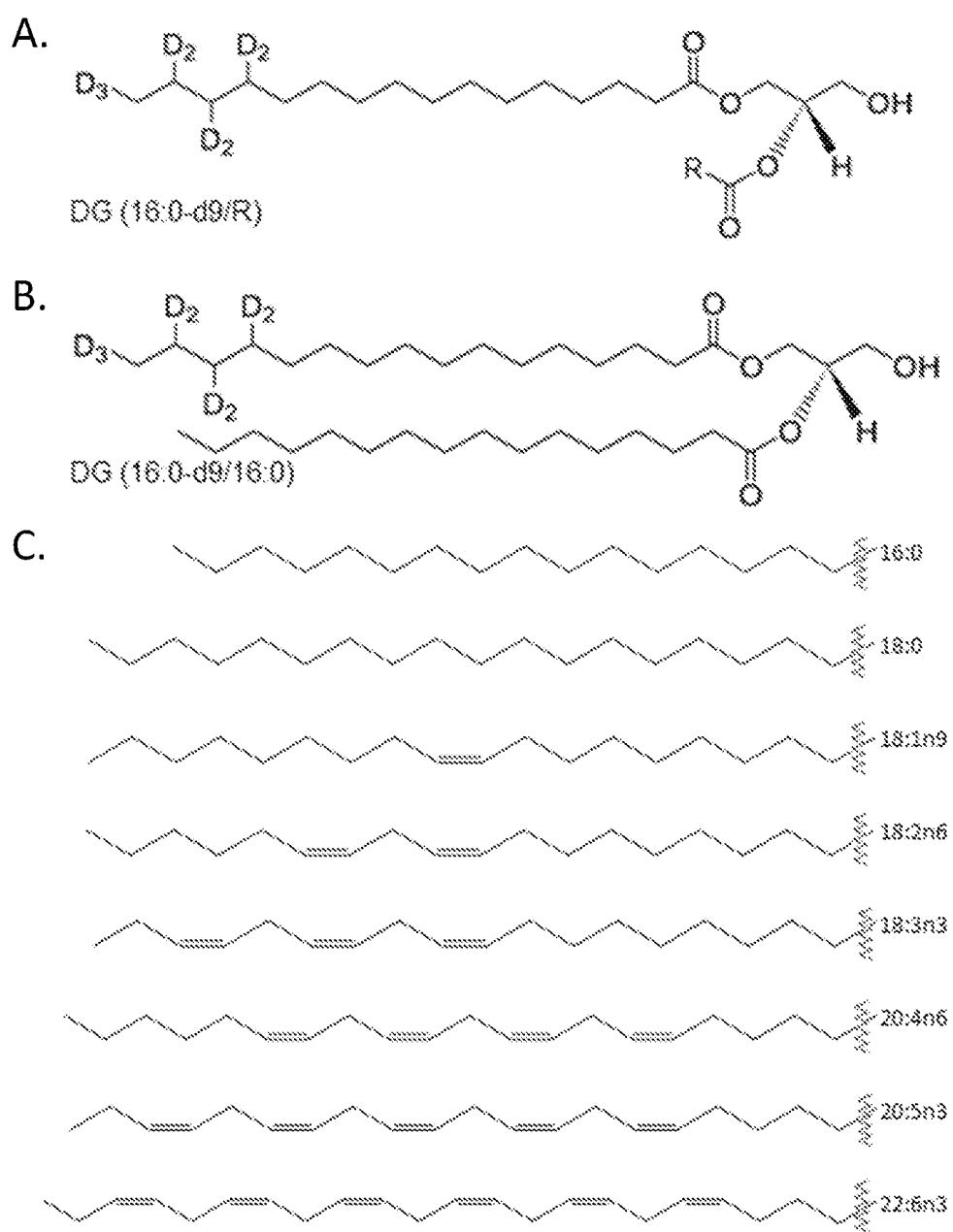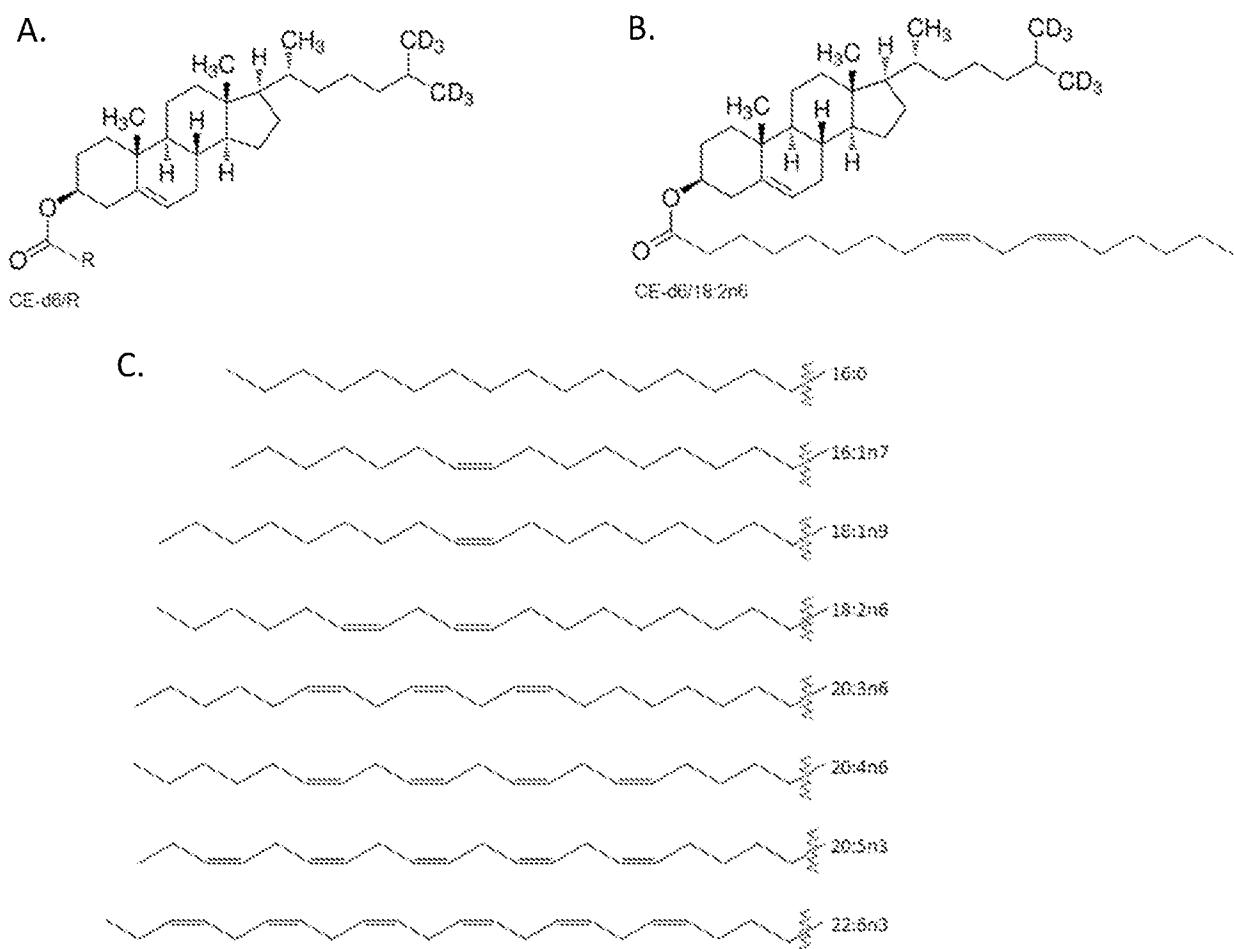
FIG. 9
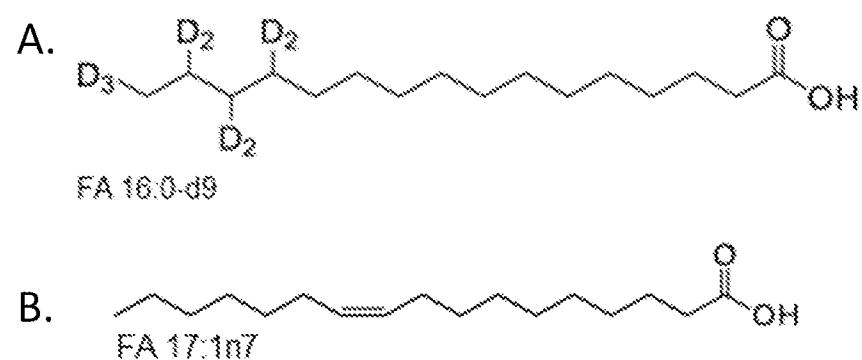
FIG. 10

FIG. 11
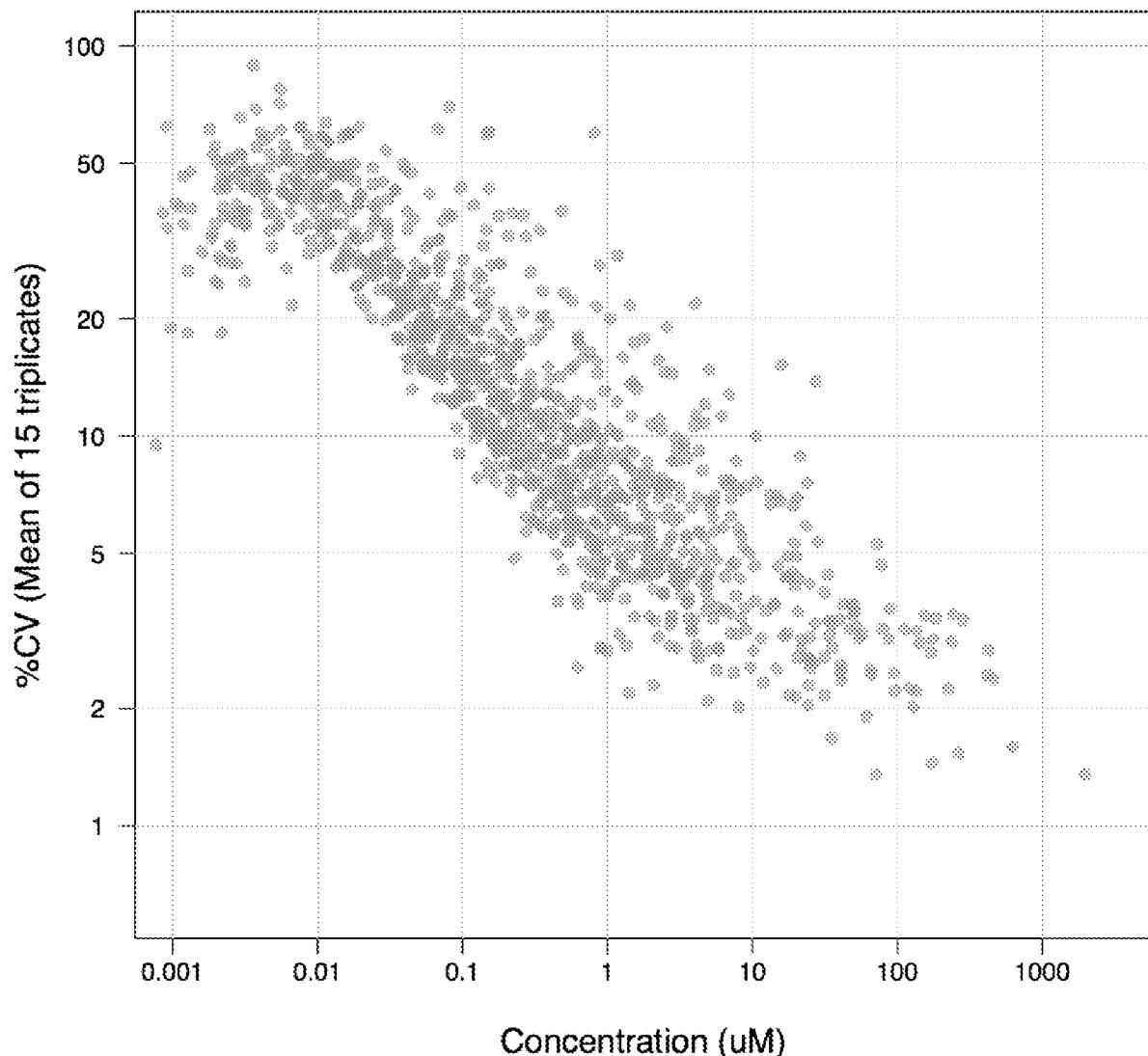
FIG. 12
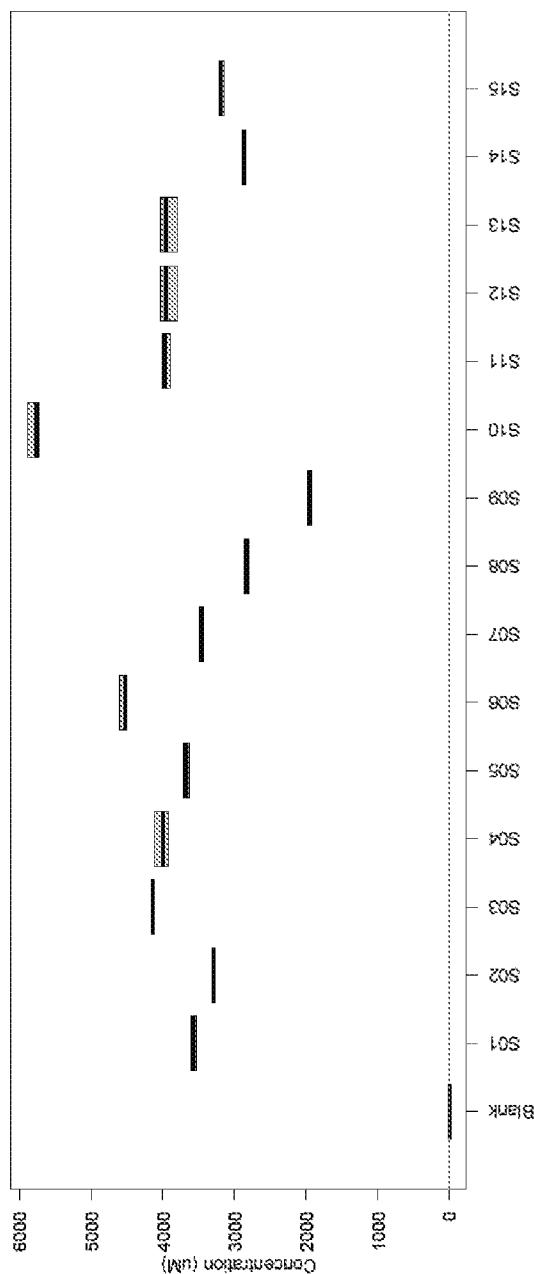
FIG. 13
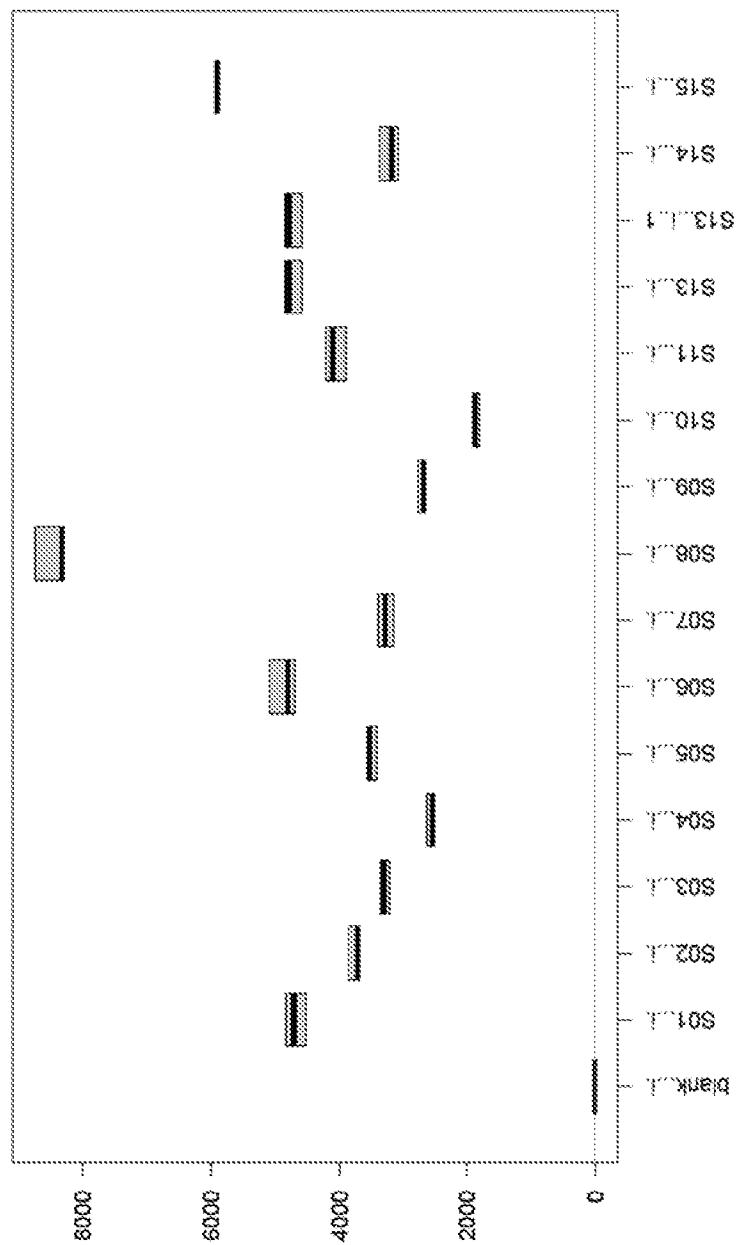
FIG. 14
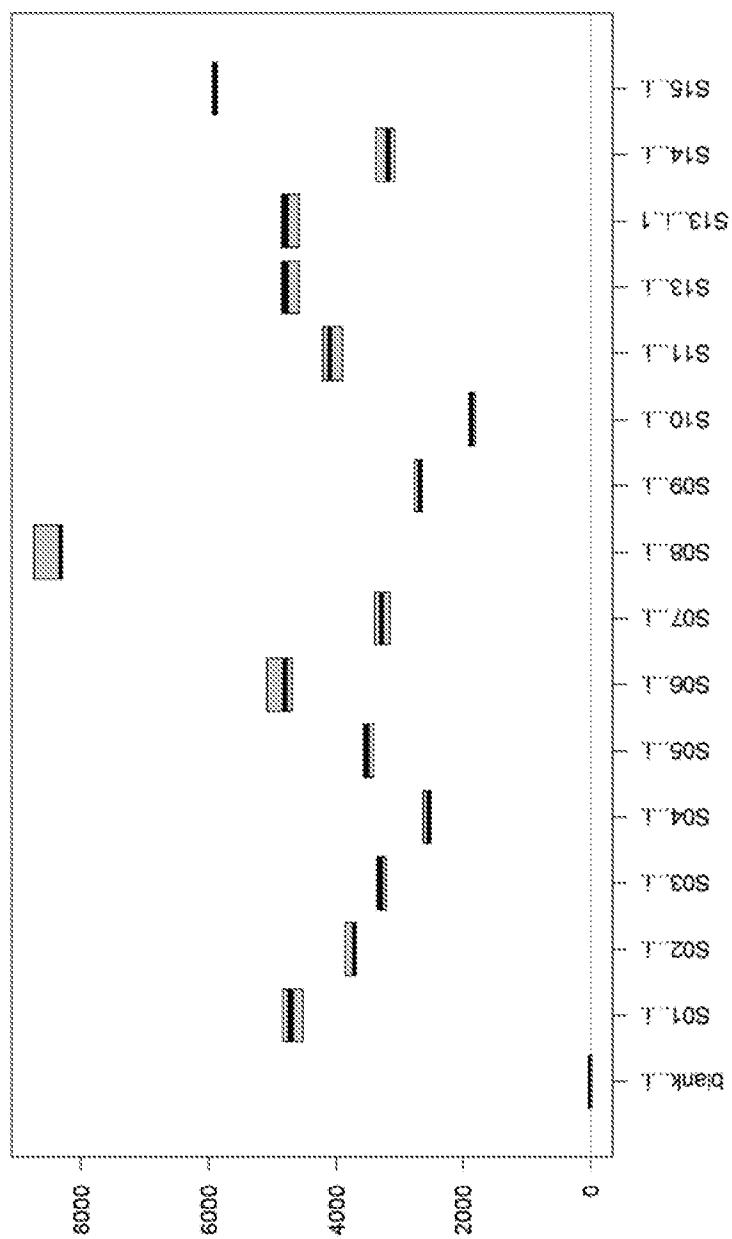
FIG. 15A
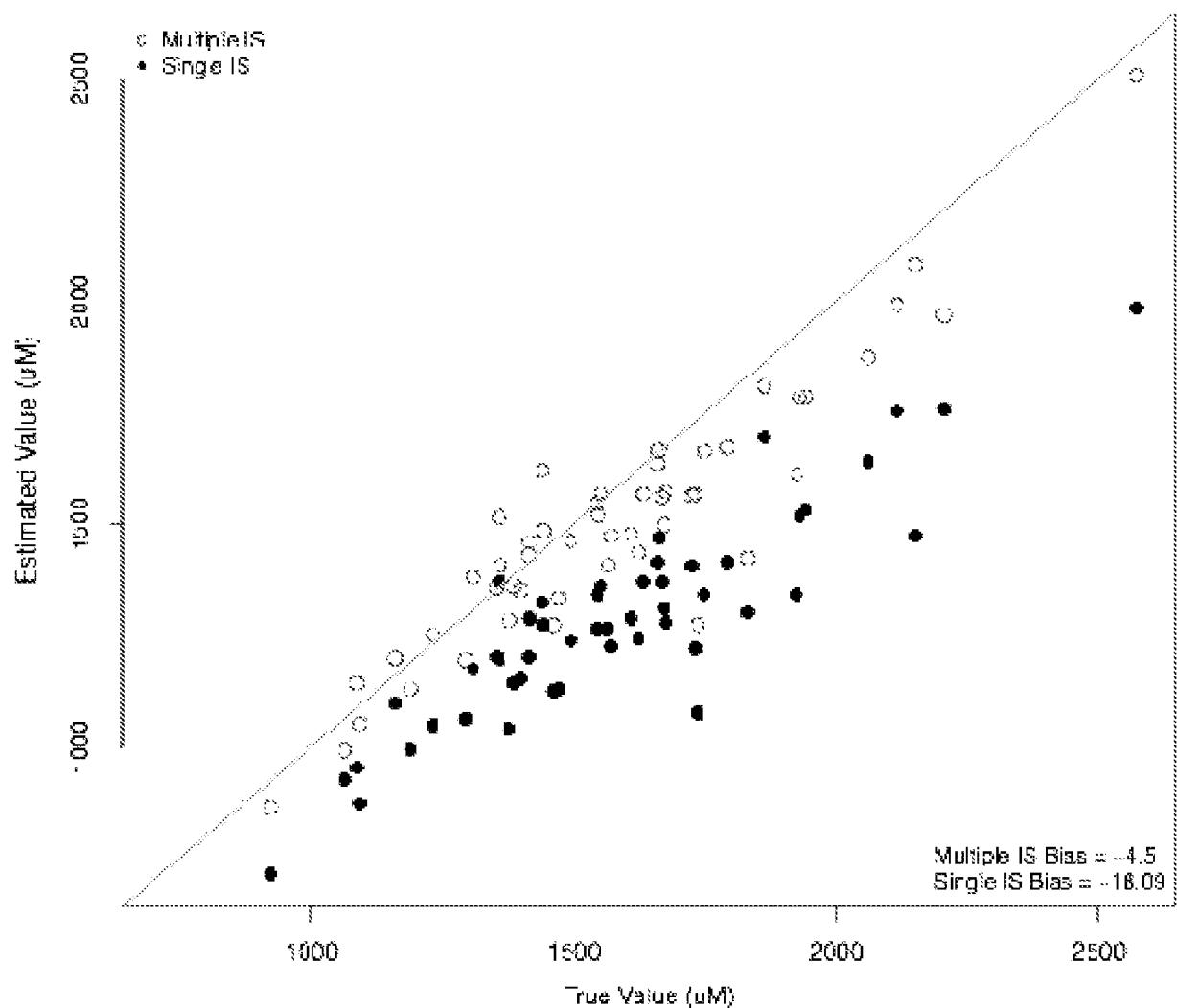
FIG. 15B
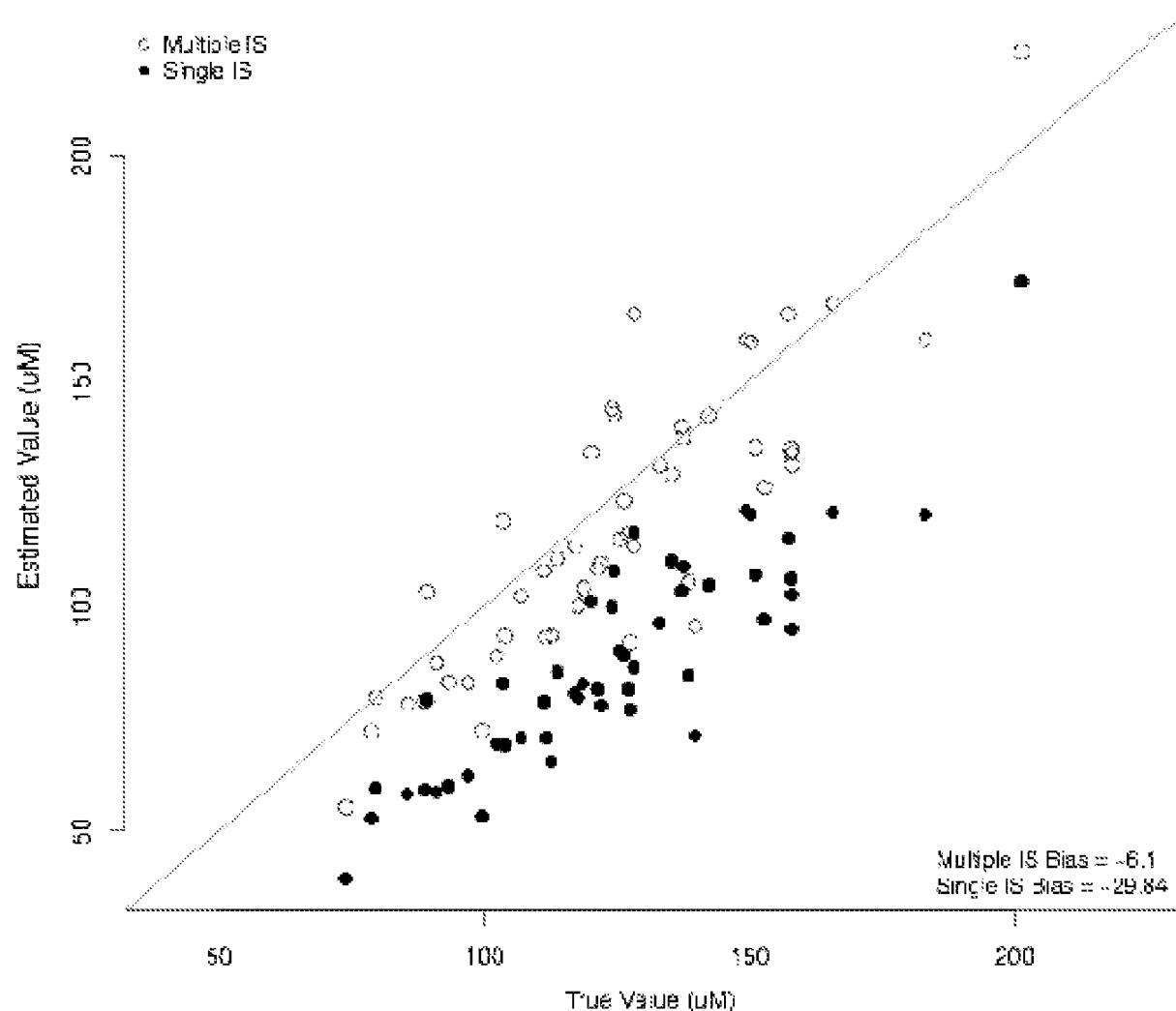
FIG. 15C
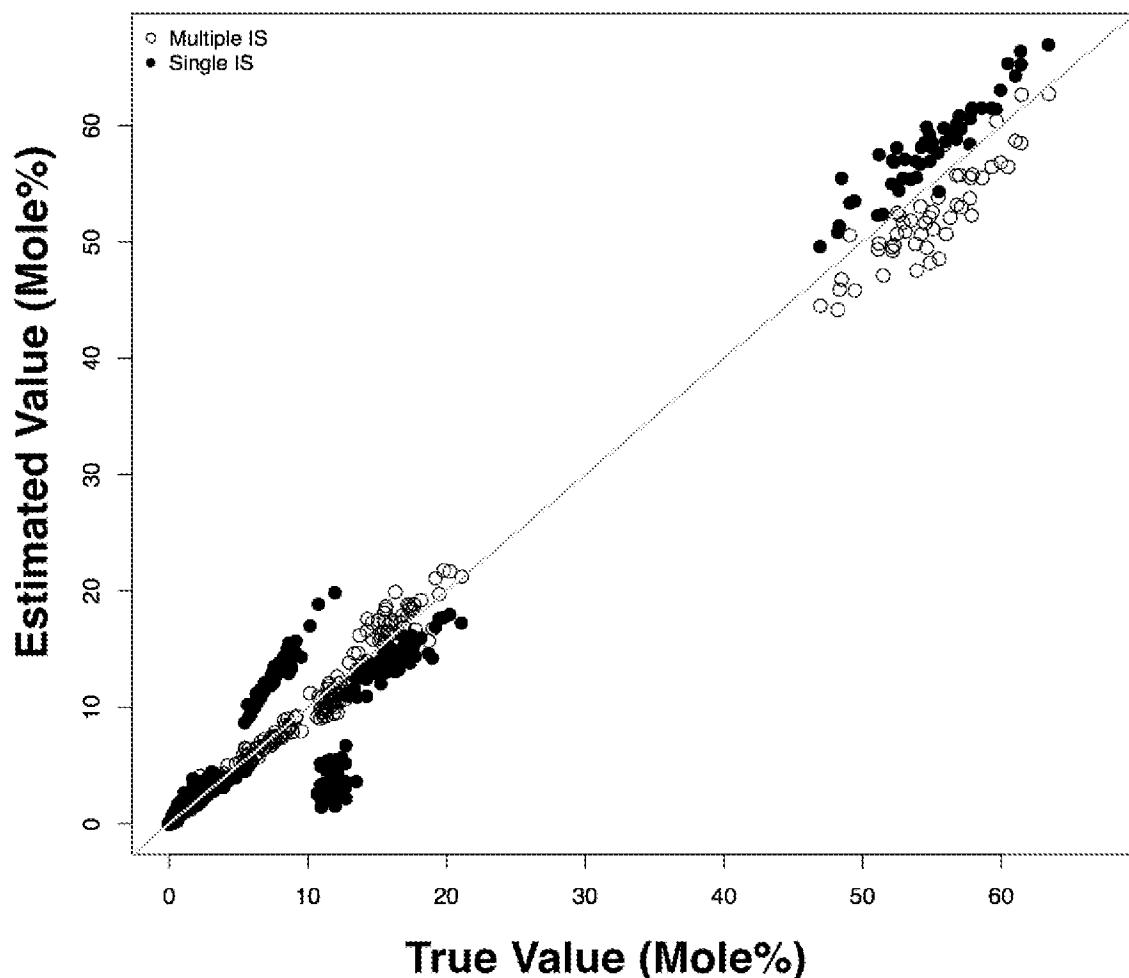
FIG. 16
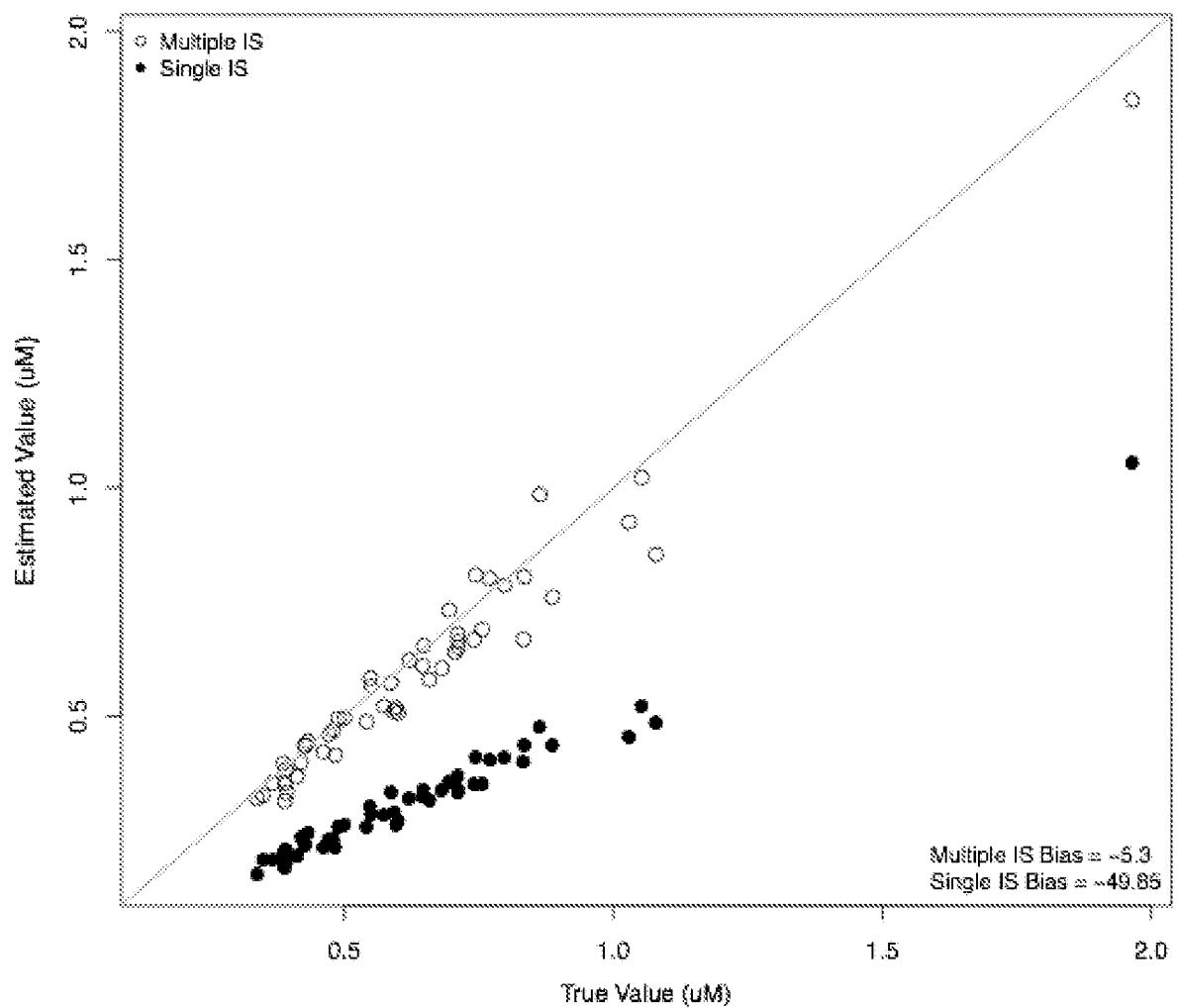
FIG. 17A
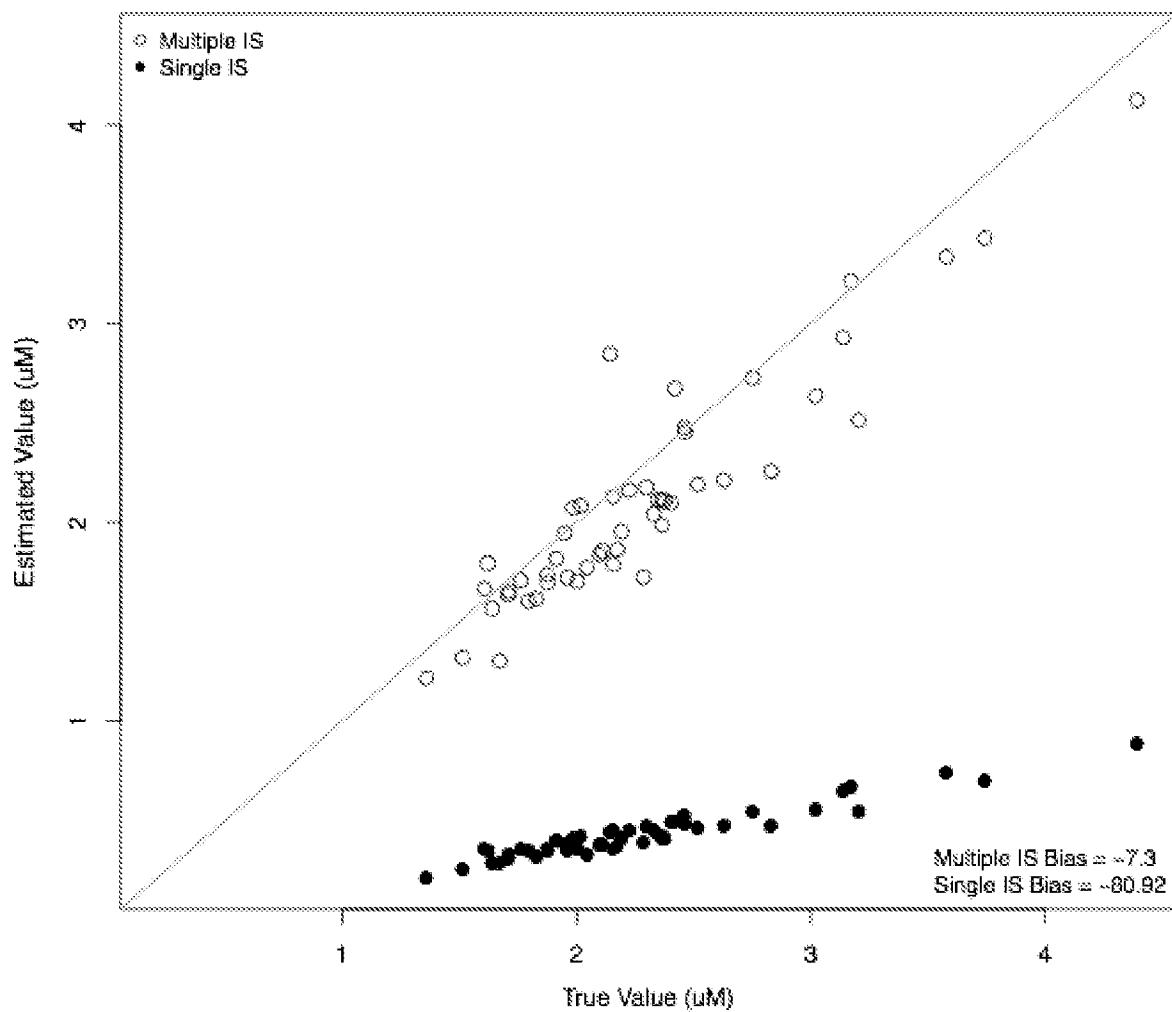
FIG. 17B
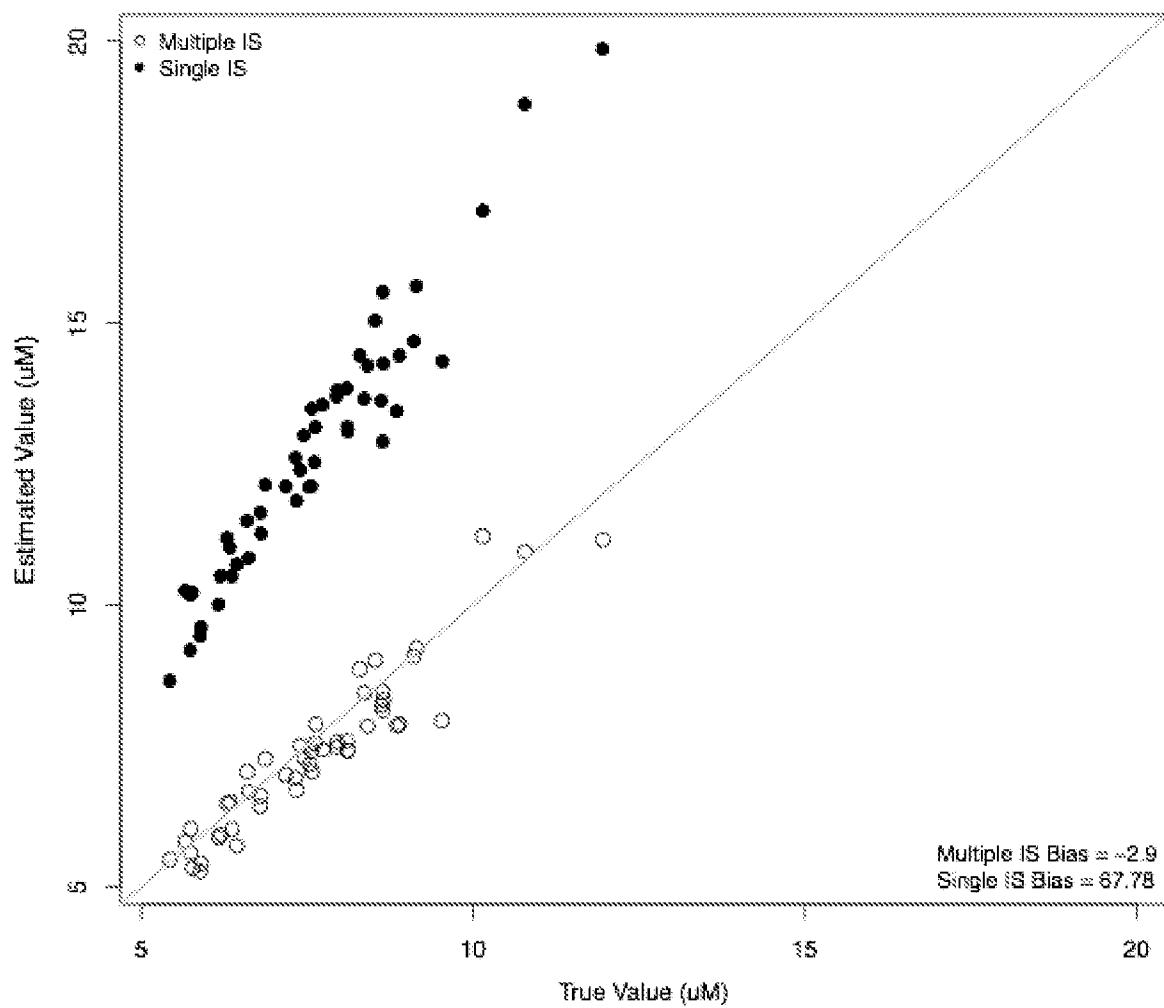
FIG. 17C
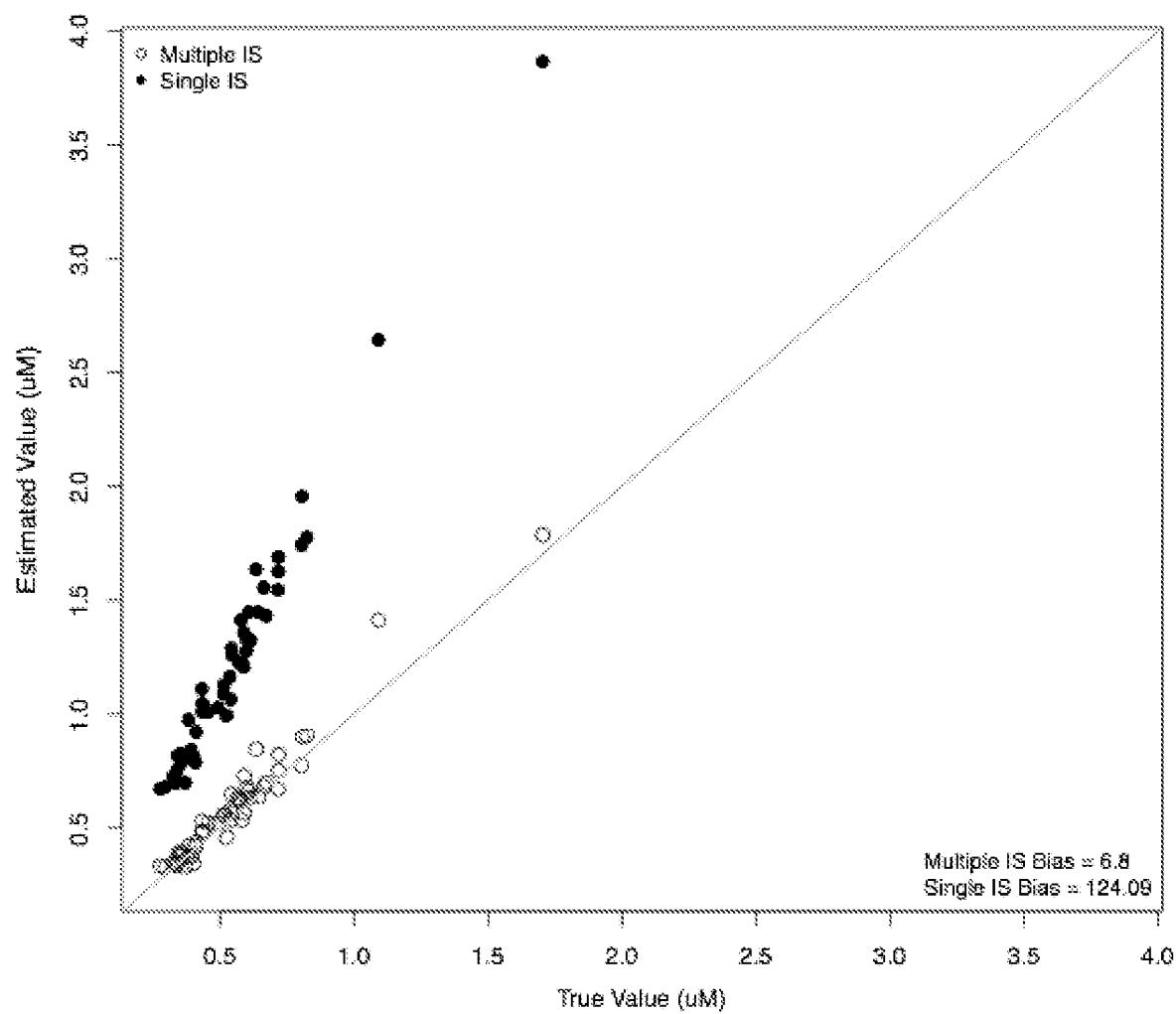
FIG. 17D

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 7/64, C12Q 1/68, G01N 33/58, G01N 33/92 (2015.01)
CPC - C12P 7/64, C12P 7/6409, C12Q 1/68, G01N 33/58, G01N 33/92, G01N 2570/00, G01N 2800/7085
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) IPC(8)- C12P 7/64, C12Q 1/68, G01N 33/58, G01N 33/92 (2015.01); CPC- C12P 7/64, C12P 7/6409, C12Q 1/68, G01N 33/58, G01N 33/92, G01N 2570/00, G01N 2800/7085
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC- 435/6.11, 435/134, 436/106, 530/391.3, 564/291; Patents and NPL (classification, keyword; search terms below)
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Pub West (US EP JP WO), Pat Base (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), Google Patent, Google Scholar, Free Patents Online; search terms: lipid, acyl, acylation, fatty, acid, label, tag

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/016586 A1 (UNIVERSITY OF DUNDEE) 30 January 2014 (30.01.2014), pg 6, ln 8 to pg 7, ln 8	1-39, 133-162, 250, 251
A	MURPHY, et al. "Mass Spectrometric Analysis of Long-Chain Lipids." Mass Spectrometry Review [online], July/August 2011 [Retrieved on 2015-09-25], Volume 30, Issue 4, Retrieved from the Internet: <DOI: 10.1002/mas.20284>, see entire document, especially Introduction; pg 2, para 1	1-39, 133-162, 250, 251
A	US 2010/0159540 A1 (RODRIGUEZ et al.) 24 June 2010 (24.06.2010), para [0006], [0024], [0050]	1-39, 133-162, 250, 251
A	US 2007/0082399 A1 (EGOROVA-ZACHERNYUK) 12 April 2007 (12.04.2007), para [0016], [0026], [0068]-[00076]	1-39, 133-162, 250, 251
A	US 2004/0063118 A1 (GROSS et al.) 01 April 2004 (01.04.2004), para [0033], [0056], [0106], [0119], [0127]-[0129], [0136]	1-39, 133-162, 250, 251

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 2015 (20.11.2015)	**2 3 DEC 2015**

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450	Lee W. Young
Facsimile No. 571-273-8300	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/42904

Box No. II   **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III   **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-39, 133-162, 250, and 251, drawn to methods for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest.

Group II: Claims 40-132, 163-249, and 252-254, drawn to compositions and kits for use as an internal standard comprising one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest.

-- Please See Supplemental Box --

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-39, 133-162, 250, 251

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

Continued from Box No. III, Observations where unity of invention is lacking.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group II does not require methods for synthesizing one or more mixtures of lipid molecules representative of the composition of lipid molecular species present in one or more corresponding lipid classes in a sample of interest, the method comprising:
attaching an isotopically-labeled fatty acid at a first position on a lipid backbone through an acylation reaction for a lipid class having at least two acyl groups; and attaching a mixture of at least two different fatty acids to the lipid backbone at a separate position through an acylation reaction, wherein the mixture of fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, attaching a mixture of at least two different fatty acids to an isotopically labeled lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group, attaching a mixture of at least two different isotopically-labeled fatty acids to a lipid backbone at a single position through an acylation reaction for a lipid class having at least one acyl group; attaching a single isotopically-labeled fatty acid to a lipid backbone at a first position through an acylation reaction for a lipid class having at least two fatty acids and attaching a mixture of at least two different fatty acids to a separate position on the lipid backbone through an acylation reaction, as required by Group I.

Shared Common Features

The only feature shared by Groups I and II that would otherwise unify the groups is an isotopically-labeled fatty acid at a first position on a lipid backbone; a mixture of at least two different fatty acids; a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids; wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest, and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest. However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by US 2004/0063118 A1 to Gross, et al. (hereinafter 'Gross'). Gross teaches an isotopically-labeled fatty acid at a first position on a lipid backbone (para [0119], [0128]); a mixture of at least two different fatty acids (para [0056], [0106], [0136]); a lipid backbone having a single isotopically-labeled fatty acid at a first position on the lipid backbone (para [0119], [0128]) and having a mixture of at least two different fatty acids at a separate position on the lipid backbone, wherein the lipid backbone is for a lipid class having at least two fatty acids (para [0033], [0056], [0106], diacyl- or triacylglycerides.) wherein the mixture of the at least two fatty acids is representative of the fatty acids that occur in the corresponding lipid class in the sample of interest (para [0119], [0129]), and wherein each of the fatty acids in the mixture is present at a ratio representative of the ratio of occurrence of the fatty acid in the lipid molecular species present in the corresponding lipid class in the sample of interest (para [0127], [0129], [0136]).

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.